

Title	Studies on a Novel Bitter Amino Acid, Pulcherrimine in the Green Sea Urchin Gonads(Dissertation_全文)
Author(s)	Murata, Yuko
Citation	Kyoto University (京都大学)
Issue Date	2001-03-23
URL	http://dx.doi.org/10.11501/3183624
Right	
Type	Thesis or Dissertation
Textversion	author

**Studies on a Novel Bitter Amino Acid,
Pulcherrimine
in the Green Sea Urchin Gonads**

Yuko Murata

2001

CONTENTS

ABBREVIATIONS ii

INTRODUCTION 1

CHAPTER I. Occurrence of bitter-tasting sea urchin *Hemicentrotus*
pulcherrimus in the sea off Iwaki 3

I-1. The frequency of occurrence of bitter gonads of the green sea urchin 4

I-2. Seasonal changes in the frequency of occurrence of bitter-tasting sea urchin ... 7

CHAPTER II. Isolation and structure elucidation of a novel bitter amino acid,
pulcherrimine, from the green sea urchin ovaries 14

II-1. Isolation from the green sea urchin ovaries 14

II-2. Chemical structure 17

II-3. Stereochemistry 22

CHAPTER III. Determination of *pulcherrimine* in the green sea urchin gonads 25

III-1. Examination of a method for analysis 25

III-2. Correlation between bitterness and content 30

III-3. Seasonal change in content 33

CHAPTER IV. Sensory tests for *pulcherrimine* 36

IV-1. Detection and recognition thresholds 37

IV-2. Thresholds in the extracts from sea urchin gonads 40

CHAPTER V. Behavioral responses to *pulcherrimine* using the CTA paradigm
in C57BL/6 and BALB/c mice 44

V-1. Behavioral thresholds in C57BL/6 and BALB/c mice 45

V-2. Behavioral responses using the CTA paradigm in C57BL/6 and BALB/c mice 48

CONCLUSION 54

SUMMARY 55

ACKNOWLEDGMENTS 58

REFERENCES 59

ABBREVIATIONS

Ac ₂ O	acetic anhydride
AcOH	acetic acid
Boc ₂ O	di- <i>t</i> -butyl dicarbonate
BuOH	<i>n</i> -butanol
COSY	correlation spectroscopy
DMF	<i>N,N</i> -dimethylformamide
EtOAc	ethyl acetate
EtOH	ethanol
HMBC	¹ H-detected multiple-bond heteronuclear multiple quantum coherence
HMQC	¹ H-detected multiple quantum coherence spectrum
MeCN	acetonitrile
MeI	methyl iodide
MeOH	methanol
MPLC	medium pressure liquid chromatography
NOE	nuclear Overhauser effect
NOSY	nuclear Overhauser and exchange spectroscopy
SD	standard deviation
SE	standard error
TEA (Et ₃ N)	triethylamine

INTRODUCTION

Sea urchin gonads are one of the most popular sea foods in Japan, due to its peculiar thick taste. The annual catch of sea urchin in Japan amounts to approximately thirteen thousand tons (FAO 1996). The gonads are eaten in a variety of ways: raw, steamed, grilled, salted and so on. The green sea urchin (*Hemicentrotus pulcherrimus*), 'Bafun-uni' in Japanese, is widely distributed among the Japanese costal areas. It is one the most important fishery products on the south-west coasts of Japan. It has long been regarded that the green sea urchin is the most suitable species as a raw material for salted sea urchin gonads. The product as such called '*Echizen Uni*' is a speciality of Fukui Prefecture (Kawana, 1938). Therefore, there is increasing interest in the reproductive biology of the green sea urchin, and there are many reports on the reproductive biology of this organism at the sea off Fukui (Kawana, 1938; Matsui, 1966).

Komata *et al.* (1962a; 1962b; 1962c; 1964) investigated the extractive components of the edible gonads of the green sea urchin, and specified the certain taste-active components in the extracts. That is, they elucidated that Gly and Ala contribute to the sweetness, Val to the characteristic bitterness of the taste of green sea urchin gonads, and Leu and Ile have a similar role as Val. However, green sea urchin of which the gonads taste extremely bitter are often found in the catch in the Tohoku area, for example, Fukushima Prefecture. Such sea urchins are not acceptable as food and have no commercial value.

Another unpleasant taste: pungent taste, which often occurs in frozen sea urchin gonads was found to be due to carbonyl compounds (Miwa 1975). These compounds are considered to be derived from amino acids by a transaminase reaction, from lipids by oxidation, or from acetal lipids by hydrolyzation during the frozen strage. It was also reported that the gonads of cultured sea urchin which are fed high protein content feed sometimes taste bitter, and the bitterness might be attributable to certain bitter amino acids formed (Hoshikawa, 1998).

Numerous substances which elicit a bitter taste have so far been determined and the structures of the bitter substances are extremely diverse. It has been considered that several receptor and transduction mechanisms are involved in the bitter taste stimulus, however, such mechanisms have not been clarified yet and the study of such mechanisms is now in progress. As to the transduction mechanisms on bitter reception, several different pathways have

recently been proposed: bitter responses occur via the inhibition of the K^+ channel, production of IP_3 , activation of gustducin, and binding of bitter substances to the lipid layers of the taste receptor membranes (Kashiwayanagi and Kurihara, 1999). On the other hand, from the view point of the utilization of bitter substances as food and/or medicine, the investigation of the mechanisms of receptor and transduction should prove to be important.

The present study was undertaken to clarify the bitter substance in green sea urchin gonads. First of all, the frequency of occurrence of the bitter-tasting sea urchins collected in the sea off Iwaki in Fukushima Prefecture was preliminarily examined. Secondly, a substance responsible for the bitterness was isolated, and subsequently, it was elucidated to be a novel sulfur-containing amino acid by determining of its chemical structure. Thus it was named pulcherrimine (Pul) after the scientific name for the green sea urchin, *Hemicentrotus pulcherrimus*. Thirdly, a method for the determination of Pul in the sea urchin gonads was devised. This method consists of the formation of dimethylaminoazobenzenesulphonyl chloride (Dabs-Cl) derivatives of Pul (dabsylation), and the separation of the dabsylate using reversed-phase high-performance liquid chromatography. Lastly, the taste quality and the threshold for Pul were examined by sensory tests by a panel, and behavioral experiments using experimental animals.

CHAPTER I

Occurrence of bitter-tasting sea urchin *Hemicentrotus pulcherrimus* in the sea off Iwaki

The green sea urchin *Hemicentrotus pulcherrimus* is widely distributed in Japan. This species of sea urchin is one of the important coastal fishery products of south-west Japan. However, this resource on the northeast coasts (i.e., Fukushima Prefecture) is hardly utilized. In the sea off Iwaki, Fukushima Prefecture, green sea urchins with bitter tasting gonads are often found and the sea urchins as such have little commercial value. In the Iwaki area, grilled sea urchin gonads called 'Kaiyaki' is a particular local favorite food. However, for Kaiyaki, the northern purple sea urchin (*Strongylocentrotus nudus*) is used instead of the green sea urchin. The bitterness of the green sea urchin gonad seems to be perceived as a pungent or an astringent taste, expressed by the word 'egumi' in Japanese.

There are some reports on unpleasant tastes of sea urchin gonads of which the causes have been ascribed to bitter amino acids and carbonyl compounds. Northern purple sea urchin fed with sand lance *Ammodytes personatus* also sometimes has bitter tasting gonads. In this case, the bitter taste has been proved to be attributable to bitter amino acids, such as valine, leucine, isoleucine, etc. (Hoshikawa et al., 1998). Miwa (1974) reported that the pungent taste of sea urchin gonads which were frozen-stored and then thawed was brought about by certain carbonyl compounds.

There are neither any studies on the occurrence of bitter substances other than amino acids or carbonyl compounds, nor any studies dealing with the frequency of the bitter tasting green sea urchin which are caught in the sea off Iwaki.

This chapter deals with the results of preliminary observations on the occurrence of green sea urchins with bitter tasting gonads inhabit at the sea off Iwaki, Fukushima Prefecture. Section 1 describes the results of duplicate preliminary examinations on the frequency of occurrence of bitter tasting green sea urchin in March 1996 and March 1997, when it is the period of maturity of this species of sea urchin. The second section details the results of tri-monthly examinations on the frequency of bitter tasting gonads during the period November 1998 to November 1999 in the same area.

I-1. The frequency of occurrence of bitter gonads of the green sea urchin

The green sea urchin is one of the edible species of sea urchin. However, in the Iwaki area of Fukushima Prefecture, this species of sea urchin is hardly the target for fisheries because of the bitter-taste of its gonads. Other than the Iwaki area, in southern Hokkaido, a similar fact that green sea urchins which have bitter tasting gonads (Agatsuma, 1992) is known.

In the first step of this study, the frequency of occurrence of bitter tasting gonads in the green sea urchin inhabits in the sea off Iwaki was examined. The bitterness of the sea urchin gonads was tested by a sensory test using a small pieces of the tissue of individual gonads. Simultaneously, the gonad extracts were analyzed for amino acids to examine the possibility of their contribution to the bitterness of the gonads.

Materials and Methods

Green sea urchin

Green sea urchins were collected from the sea off Iwaki, Fukushima Prefecture in March 1996 and March 1997, the number of specimens collected were 94 and 99, respectively. Their gonads were dissected out, after the size and the weight were measured. The gonad index (GI) of each sample was calculated by the follows equation:

$$\text{GI (\%)} = (\text{gonad weight} / \text{body weight}) \times 100$$

Preparation of extracts

The gonads from individuals, after a small piece of the gonad tissue was separated for the sensory test of the bitterness, were separately homogenized with three volumes of 80% aq EtOH. The residue was re-extracted twice with three volumes of 80% EtOH. The homogenate was centrifuged at 1700×g for 10 min. The combined 80% EtOH extracts were evaporated to a small quantity and partitioned between water and diethyl ether. The aqueous phase was concentrated to 5 mL under reduced pressure.

Free amino acid analysis

Free amino acid analysis was performed with a Hitachi L-8500A amino acid analyzer

(Murata *et al.*, 1998).

Brief sensory test

The gonads were briefly tested for bitterness by tasting small tissue pieces (20-30 mg) taken from the gonads (this test is called a brief sensory test for convenience). The brief sensory test was carried out by three experienced subjects.

Results and Discussion

Table I-1 shows the frequency of occurrence of bitter gonads in the green sea urchins collected from the sea off Iwaki, Fukushima Pref. in March 1996. All specimens were judged to be mature and their sex was readily distinguished by the oozed gametes (Fuji, 1960). Male and female sea urchins were almost identical in the biological data such as test diameter, test height, body weight, gonad weight and gonad index. Ninety-five percent of the female individuals had bitter ovaries, while none of the male individuals had bitter testes. In March 1997, all of the individuals of which the gonads gave a bitter taste were found to be female. These results suggest that the bitterness of the green sea urchin is specific to the mature ovaries. The free amino acid (FAA) analysis of both male and female gonads, showed that there is no significant difference in the contents of bitter amino acids such as Val, Ile, Leu between the non bitter testes and the bitter ovaries (Table I-2). This fact ruled out the possibility of the participation of the bitter amino acids being the case of the bitterness of the sea urchin ovaries. The bitterness was perceived in the water soluble fraction, instead of the ether fraction. Therefore, another possibility that the bitter principle may be due to carbonyl compounds was denied, because such carbonyl compounds are soluble in ether.

Table I-1. The frequency of occurrence of bitter gonads in green sea urchins together with biological data

	Male	Female
Number of specimens (A)	50	44
Bitter gonad (B)	0 (0%) [†]	42 (95%) [†]
Test height (mm) ^{†1}	20.0 ± 2.7	19.7 ± 2.8
Test diameter (mm) ^{†1}	37.9 ± 3.8	37.6 ± 4.0
Body weight (g) ^{†1}	22.1 ± 1.1	21.7 ± 6.4
Gonad weight (g) ^{†1}	2.4 ± 1.1	2.5 ± 1.0
Gonad index (%) ^{†1,†2}	10.8 ± 4.5	11.2 ± 4.5

[†] Number of specimens having bitter gonads. Values in parentheses are frequency (B/A × 100%).

^{†1} Mean ± standard deviation.

^{†2} Gonad weight / body weight × 100.

Table I-2. Free amino acids in the extracts of the sea urchin gonads (mg/100g)[†]

	Testis	Ovary
Glutamic acid	102.8 ± 8.4	43.4 ± 15.0
Glycine	779.2 ± 136.4	1019.2 ± 209.3
Alanine	151.8 ± 58.3	94.4 ± 22.1
Valine	42.4 ± 39.8	36.2 ± 30.9
Methionine	19.0 ± 12.2	17.4 ± 12.1
Isoleucine	29.8 ± 26.3	32.4 ± 23.8
Leucine	46.8 ± 48.8	41.6 ± 38.5
Tyrosine	35.7 ± 24.5	37.8 ± 25.5
Phenylalanine	29.8 ± 27.5	29.5 ± 23.8
Tryptophan	15.8 ± 13.8	9.8 ± 5.7
Arginine	50.2 ± 40.5	91.9 ± 34.4
Proline	37.3 ± 21.5	17.1 ± 8.1
Total FAA	1632.5 ± 150.3	1723.4 ± 152.1

[†] Mean ± SD (n=5).

I-2. Seasonal changes in the frequency of occurrence of bitter-tasting sea urchin

In the preceding study (Section I-1), the frequency of occurrence of bitter gonad in the green sea urchin was examined in March 1996 and March 1997. The results indicated that the bitterness of the green sea urchin gonads was specific to mature ovaries. In March (1996 and 1997), all the individuals collected from the sea off Iwaki were mature. Fuji (1960) divided the development process of the gonads of the two species of sea urchins, *Strongylocentrotus nudus* and *S. intermedius*, into six stages by histological and anatomical observations. Matsui (1966) and Ito (1989) found that green sea urchin in Fukui Prefecture and Saga Prefecture has a clearly defined annual reproductive cycle.

In this study, tri-monthly examinations were carried out to clarify the seasonal changes in the maturity and the frequency of occurrence of bitter tasting gonads of green sea urchins inhabit in the sea off Iwaki in Fukushima Prefecture.

Materials and Methods

Green sea urchins

At intervals of every three months from November 1998 to November 1999, 100 green sea urchins (*H. pulcherrimus*) were randomly collected from the sea off Iwaki, Fukushima Prefecture. This sampling will here after be called tri-monthly sampling for convenience. As soon as the specimens collected were carried to the laboratory, the biological factors were measured and the gonads were dissected out as described in the preceding section. The gonad index (GI) of each individual was also calculated by the same equation as mentioned in the preceding section:

$$\text{GI (\%)} = (\text{gonad weight} / \text{body weight}) \times 100$$

Determination of sex and maturity

In this study, the sampled green sea urchins were divided into mature and immature individuals. Mature individuals were defined in this study as those with gametes which ooze from the gonads. Immature individuals were defined as those with gametes which do not ooze from the gonads. The sex of the mature individuals was identified from the oozed gametes

(Fuji 1960).

Histological observations

Four to seven immature individuals collected every three months were histologically observed in order to determine the stage of their gonadal development. Small pieces of gonad from each individual were fixed in Bouin's solution, embedded in paraffin, and sectioned at 6 μ m thick. The sections were stained with hematoxylin and eosin, and then observed with an optical microscope to determine the sex and gametogenic stage of the gonads. The gonadal maturity of each individual was assessed according to the stage classification initially proposed by Fuji (1960) modified slightly by Unuma *et al.* (1996). The stage classification is as follows:

Stage 0 (neuter): No obvious germ cells are observed. Sexes are unidentified.

Stage 1 (developing virgin or recovering spent): A few young oocytes or small clusters of spermatogenic cells are present along the inside of the follicle walls. Nutritive phagocytes occupy the follicular lumens.

Stage 2 (growing): Follicle walls are lined with oocytes or spermatocytes. The center of the lumens are still filled with nutritive phagocytes.

Stage 3 (pre-mature): In the center of the lumen, nutritive phagocytes are replaced with a number of oocytes or small masses of spermatozoa.

Stage 4 (mature): Follicular lumens are filled with large numbers of mature eggs or spermatozoa. Nutritive phagocytes are recognized only in the periphery of follicle.

Stage 5 (spent) : Follicular lumens are almost empty with a few relict eggs or small masses of relict sperm.

Preparation of extracts

Every gonad was separately homogenized with an equal volume of water, after a small piece of the gonad tissue was separated for the sensory test. The homogenates were heated at 100°C for 12 min. After being cooled, the homogenate was centrifuged at 1700×g for 10 min. Fifty microliters of the supernatant were subjected to the judgement of bitterness, and the remainder was transferred to a volumetric flask (10 mL or 50 mL). The precipitate was re-extracted twice, first with two volumes and then one volume of water, respectively, in the same manner as above. The extracts were combined in the volumetric flask and filled up to the mark with distilled water and were frozen-stored below -30°C until use.

Sensory tests

Firstly, the gonads were applied to a brief sensory test by the same manner as Section I-1. After a brief test, all extracts of the gonads was examined for the bitterness by three experienced subjects.

Statistical analysis

The GI of the tri-monthly samples between November 1998 and November 1999 were statistically analyzed. The analysis was performed by the *F*-test for the difference of the variance in each month. When the variance was equal between tri-monthly samples, the *t*-test was applied, while when the variance was different, the Cochran-Cox test (Wakabayashi, 1984) was applied.

Results

The frequency of occurrence of mature male, female, and immature sea urchins

Table I-3 shows the frequency of mature male, female, and immature green sea urchins. In November 1998 and February 1999, all of the sea urchins examined were mature, and thus the sex of each individual was easily distinguished. The frequency of female sea urchins was 40%. That of the male sea urchins was, naturally, 60%. In May and August 1999, the frequency of immature individuals was relatively high: 20% and 60%, respectively. Seven specimens from each sample out of the immature individuals collected in May and August 1999 were subjected to a histological observations. These observations revealed that these individuals were at the recovering spent stage (Fuji, 1960). The frequency of mature males was 50% and 20%, in May and August, respectively, and that of mature females was 30% and 20%, respectively. In November 1999, 56% of individuals were identified as male and 40% as female. Four immature individuals were identified as females by microscopic observations: two of them were judged to be at the growing stage, and the other two at the pre-mature stage.

Test diameter, test height, body weight

The mean test diameter, test height and body weight of individuals were approximately the same through the observations for the five months, i.e. 22 mm, 41 mm and 23 g, respectively (Table I-4).

Table I-3. The frequency of occurrence of mature male and female, and immature sea urchin[†]

	Mature		Immature ^{††}
	Male	Female	
November 1998	56	44	0
February 1999	57	43	0
May 1999	50	30	20
August 1999	20	21	59
November 1999	56	40	4

[†] As the figures express the number of relevant specimens out of 100 specimens collected at the same time, they mean the percentage of cases.

^{††} Gametes did not ooze from the gonads and the sex identification was impossible.

Table I-4. Test height, test diameter and body weight of green sea urchin from November 1988 to November 1999

	Month	Test height (mm) [†]	Test diameter (mm) [†]	Body weight (g) [†]
Mature male	Nov 1998	22.1±2.0	41.6±2.8	21.1±4.9
	Feb 1999	22.5±2.8	41.3±3.9	24.9±4.5
	May 1999	23.4±2.8	43.3±4.1	26.5±8.7
	Aug 1999	22.1±2.2	40.6±3.2	23.2±5.3
	Nov 1999	21.7±1.8	41.6±2.9	23.6±5.6
	mean±SD	22.4±2.4	41.8±3.4	23.9±5.9
Mature female	Nov 1998	22.1±1.6	41.9±3.3	21.6±4.5
	Feb 1999	22.3±1.6	40.9±2.8	23.4±4.5
	May 1999	21.8±1.4	41.2±2.1	22.3±3.2
	Aug 1999	21.0±2.0	39.0±3.4	20.8±4.7
	Nov 1999	21.9±1.6	40.9±2.6	22.5±4.5
	mean±SD	21.9±1.6	41.0±2.8	22.3±4.3
Immature	May 1999	23.5±2.7	43.4±3.3	27.0±6.7
	Aug 1999	22.1±2.0	40.9±3.0	23.5±5.7
	Nov 1999	22.3±0.1	40.5±0.1	24.2±1.6
	mean±SD	22.4±2.1	41.3±3.0	24.2±5.9

[†]Mean ± SD.

Seasonal changes in the gonad index

Seasonal changes in the GI of each sex of the sea urchin are shown in Fig. I-1. The GI values varied considerably among the mature specimens in each season. The mean GI values of the mature male and female gonads decreased significantly from February to May, and significantly increased from May to August. This fact suggests that spawning occurs from February to May. The GI was smallest in May for both sexes. It is equally true in the both

sexes. The GI of the immature specimens in May, August and November 1999 was 8.3 ± 0.8 (%), 12.7 ± 0.5 (%) and 7.5 ± 1.0 (%) (mean ± SE). These values were lower than that of the mature specimens.

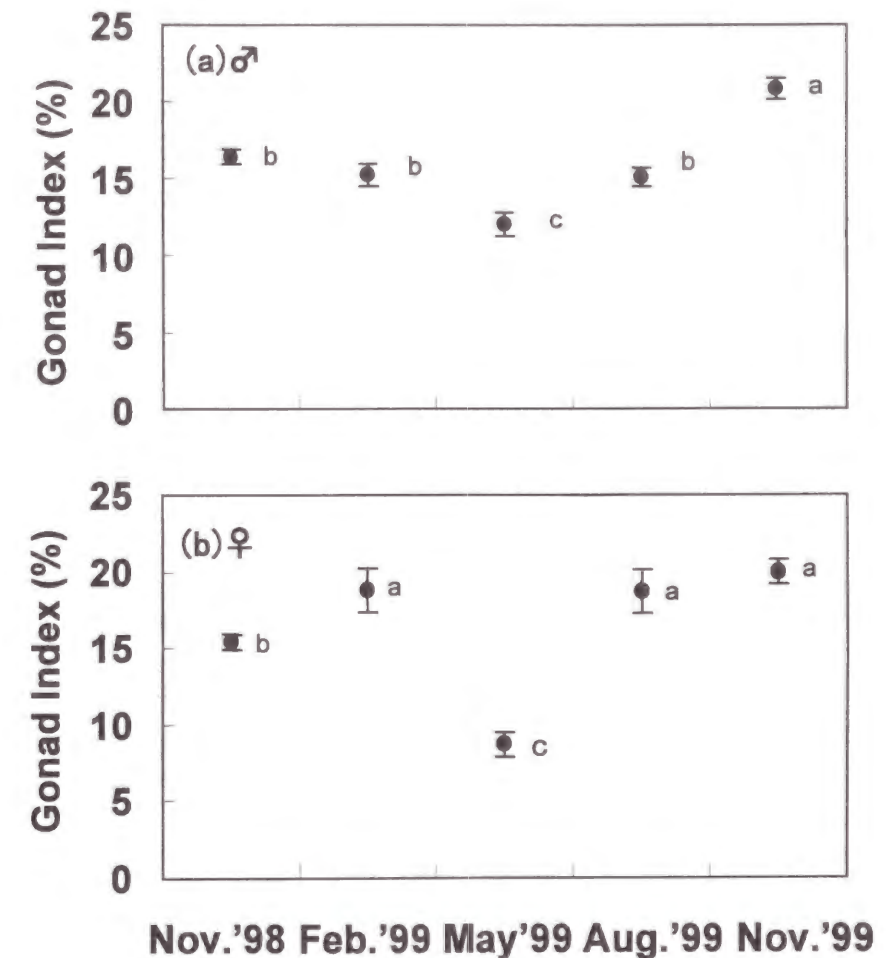


Fig. I-1. Seasonal changes in the gonad index (mean ± SE) of (a) mature male and (b) mature female of green sea urchin inhabits in the sea area off Iwaki. Among the supplementary letters, a, b, c, the mean values are significantly different from each other ($P < 0.05$).

The frequency of occurrence of bitter tasting gonads of the green sea urchin in the different seasons

Figure I-2 shows the frequency of occurrence of bitter gonads in each season. More than

95% of the ovaries of sea urchin collected in November 1998, February 1999 and November 1999 tasted bitter. On the other hand, in May and August 1999, 60% of the mature ovaries were bitter. Immature gonads had no bitter taste in May and November, but 20% of the immature gonads in August 1999 tasted bitter.

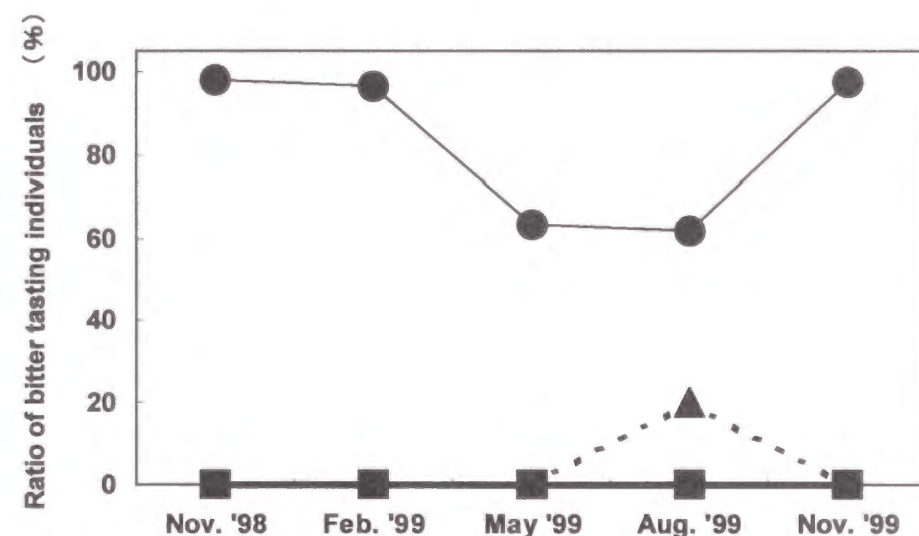


Fig. I-2. Frequency of bitter tasting gonad observed at intervals of three months; mature males (■), mature females (●) and immature individuals (▲).

Discussion

The maturation mode of green sea urchins which inhabit in the sea off Iwaki was examined based on the frequency of occurrence of mature individuals and the variation in GI throughout the period from November 1998 to November 1999. The fact that the GI values showed a large variation among the mature specimens in each season indicates that the maturation process of the sea urchins might vary among individuals. The mean GI values of gonads were observed to decrease significantly during the period between February to May 1999, were at the lowest values in May 1999, and increase thereafter. This change in the GI suggests that the major spawning season is in the period from February to May.

In May and August 1999, many immature sea urchins were observed, but, simultaneously, many mature female and male individuals were also observed. This suggests that mature individuals occur in all seasons in the sea off Iwaki. From the variation of the individual GI values and the presence of mature individuals in all seasons, the reproductive cycle of this species of sea urchin seems to be unclear, and the occurrence of the mature stage extends over a long term, at least, in this sea area.

The maturation and subsequent spawning behavior are generally considered to be controlled by the sea water temperature (Agatsuma, 1992). Ito *et al.* (1989) described for the green sea urchin that a temperature depression after a high water temperature period accelerates the maturation of gonads. In the sea area off Saga Pref., it is observed that spawning starts when the water temperature drops to 15°C. In the sea off Iwaki, the water temperature remains over 20°C from August to October. In September, the water temperature reaches its highest value and then declines. From December to June, the water temperature is usually below 15 °C (Monthly Ocean Report, Dec. 1999). Therefore, the variation of the maturation process and the long term maturation period among individual sea urchins in the sea off Iwaki may be due to the relatively low water temperature.

The frequency of occurrence of bitter ovaries accounted for more than 95% of the total examined in November 1998, February 1999 and November 1999, and 60% of the total mature ovaries, even in May and August 1999. From these results, the seasonal change in the frequency of occurrence of bitter ovaries agreed well with that of the distribution of mature ovaries.

CHAPTER II

Isolation and structure elucidation of a novel bitter amino acid, pulcherrimine, from the green sea urchin ovaries

At the sea off Iwaki, Fukushima Prefecture, green sea urchins with bitter-tasting gonads are often found. In the preceding study (Section I-1), the frequency of occurrence of the bitter-tasting sea urchin was examined. The results indicated that the bitterness of the green sea urchin gonads was specific to the mature ovaries. The bitter taste of the sea urchin ovaries has been thought to be due to the presence of such free amino acids as valine, leucine and isoleucine. (Hashimoto, 1965; Fuke *et al.*, 1991). Free amino acid contents are not significantly different between ovaries and testes. Thus, the bitter principle was predicted to differ from that of the bitter amino acids mentioned above (Section I-1).

The present Chapter describes isolation and structure elucidation of the bitter substance from the green sea urchin ovaries.

II-1. Isolation from the green sea urchin ovaries

The preceding study showed that the bitterness of the green sea urchin gonads was specific to mature ovaries. This section describes the isolation of pulcherrimine (Pul) from green sea urchin ovaries using bioassay (sensory test)-guided fractionation.

Materials and Method

Green sea urchin

Green sea urchins were collected from the sea off Iwaki, Fukushima Prefecture, during the period from March to June in 1997 and 1998, when most of the gonads were mature. After dissection, the ovaries were stored at -84C until used.

Apparatus

HPLC separations were performed with a Shimadzu LC-10ADvp liquid chromatograph

equipped with a Shimadzu RID-6A refractive index detector, a YMC pack R&D ODS column, and a Reodyne injector.

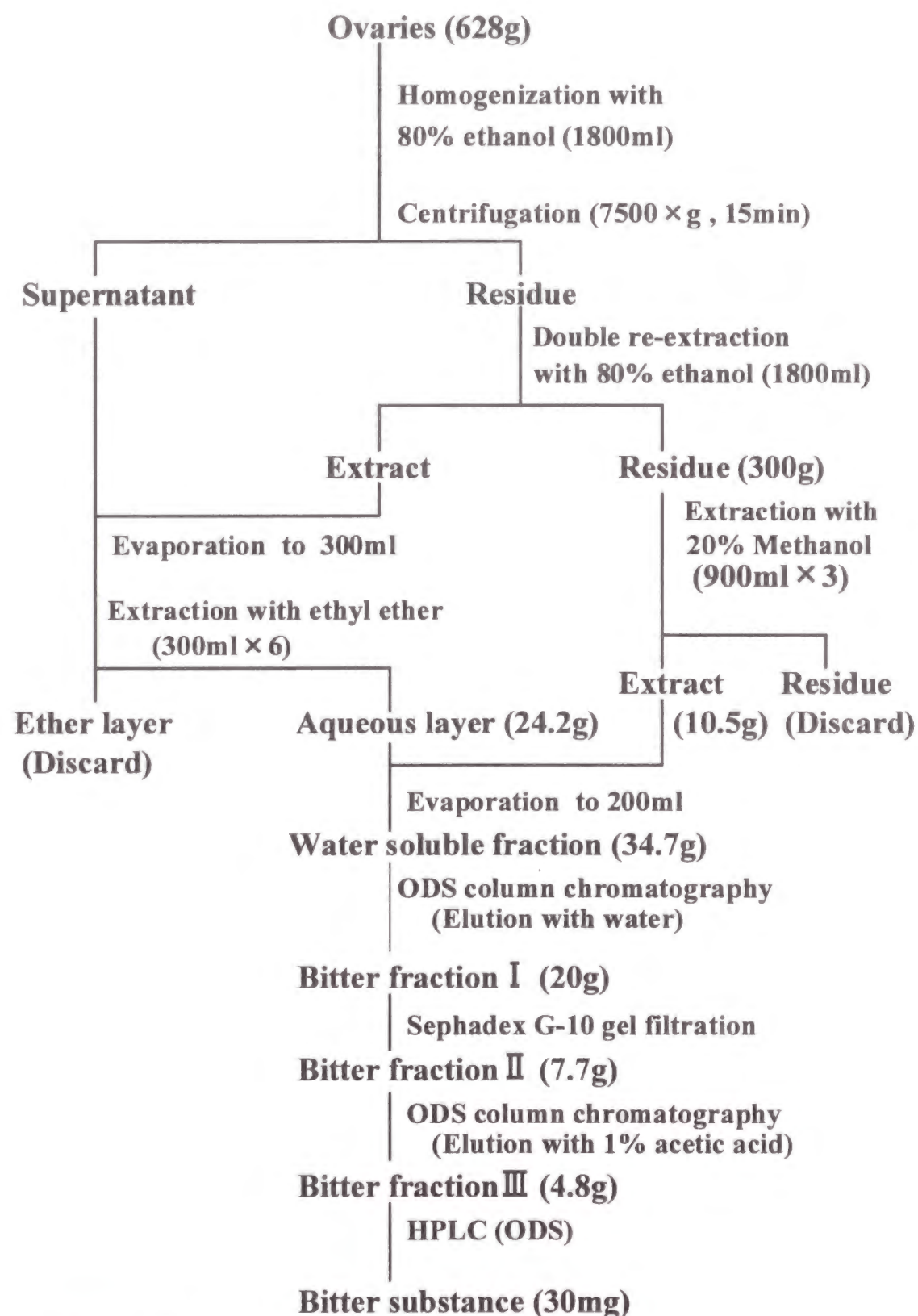
Analytical TLC was carried out on MERCK Kieselgel plates 60F₂₅₄ in 0.25mm thick. Chromatograms were visualized by either ninhydrin or sulfuric acid.

Extraction and Isolation

Frozen ovaries (628g) were homogenized and extracted with 80% aq EtOH (1.8 L × 3). The homogenate was centrifuged at 7,500 g for 15 min. The pellet was further extracted with 20% aq MeOH (0.9 L × 3). The 80% aq EtOH extracts were evaporated and partitioned between water and diethyl ether. The aqueous phase and 20% aq MeOH extracts were combined and concentrated under reduced pressure. Isolation of Pul was carried out by monitoring the bitterness using a brief sensory test. The taste of a small quantity of the solutions was determined by the authors. When fractions were 1% AcOH solutions, each solution was evaporated to eliminate AcOH and dissolved in equal volume of distilled water for sensory tests. The residue (34.7 g) was chromatographed on an ODS column (Cosmosil 140C₁₈-prep, 50 × 500 mm) with distilled water. The active fractions were separated by gel filtration on Sephadex G-10 (Amersham Pharmacia Biotech Co.Ltd., 26.4 × 1000 mm) with distilled water. The bitter fraction (7.7 g) was fractionated by ODS column chromatography (Cosmosil 140C₁₈-prep, 30 × 800 mm) with 1% aq AcOH. The active fractions were combined (4.8 g) and purified by preparative HPLC on a YMC- Pack R&D ODS column (20 × 250 mm) with 1% AcOH (flow rate, 5 mL/min) monitoring with a refractive index detector to yield a bitter principle [30.0 mg, 4.8 × 10⁻³ % based on wet weight; TLC on silica gel, R_f 0.12 (*n*-BuOH/AcOH/H₂O, 4:1:2)] as amorphous white powder (Scheme II-1).

Results and Discussion

The sea urchin ovaries (628 g wet weight) were extracted with 80% aq EtOH and then with 20% aq MeOH. The 80% aq EtOH was partitioned between water and diethyl ether. The aqueous layer and 20% aq MeOH extract were combined and separated by ODS flash chromatography, followed by gel-filtered on Sephadex G-10 with H₂O. Bitter fractions were further fractionated by MPLC and purified by HPLC on ODS with 1% aq AcOH to yield Pul (30.0 mg) as amorphous white powder.



Scheme II-1

II-2. Chemical structure

In the preceding section, a bitter principle (Pul) was isolated from the green sea urchin ovaries. Subsequently, this section describes the chemical structure elucidation of pulcherrimine (1) by spectroscopic and chemical methods. (The stereochemistry of 1 is described in the next section (Section II-3)).

Materials and Methods

Apparatus

ESI-MS and FABMS were recorded with a Micromass QUATTRO II and a JEOL JMX-SX102 mass spectrometer using thioglycerol as matrix, respectively. 1D and 2D NMR spectra were recorded either on a JEOL JNM-α600 (600MHz for ^1H , 125 MHz for ^{13}C) or a JEOL GX-270 (270 MHz for ^1H , 67.5 MHz for ^{13}C) NMR spectrometers at 300K. ^1H chemical shifts were referenced to residual solvent peaks: TMS (δ_{H} 0.00) in CDCl_3 (internal standard), CD_2HOD (δ_{H} 3.30) in CD_3OD and D_2O (internal standard). ^{13}C chemical shifts were referenced to solvent peaks: δ_{C} 77.0 in CDCl_3 , δ_{C} 49.0 in CD_3OD and D_2O (internal standard). The distortionless enhancement by polarization transfer (DEPT) experiments were performed using a transfer pulse of 135° to obtain positive signals for CH and CH_3 and negative ones for CH_2 groups. ^1H - ^1H COSY, HMBC and HMQC experiments were performed using conventional pulse sequences (Bax *et al.*, 1986; Summers *et al.*, 1986).

Optical rotation was measured on a JASCO DIP-371 digital polarimeter.

Analytical TLC was carried out on MERCK Kieselgel plates 60F₂₅₄ in 0.25mm thick. Chromatograms were visualized by either ninhydrin or sulfuric acid.

Determination of a planer structure of 1

A planer structure of 1 (Fig. II-1) was determined by HRFABMS data, and 1D and 2D NMR spectral data.

Pulcherrimine (1): $[\alpha]_{\text{D}}^{24}$ -16.5° (c 0.20, H_2O); TLC on silica gel, R_f 0.12 ($n\text{-BuOH}/\text{AcOH}/\text{H}_2\text{O}$, 4:1:2); ^1H NMR in $\text{D}_2\text{O}/\text{CD}_3\text{OD}$ (40:1) at 600MHz δ 4.19 (1H, dd, $J=6.7, 3.8$ Hz, H2'), 3.64 (1H, dd, $J=12.7, 3.1$, H2), 3.49 (1H, ddd, $J=13.1, 4.2, 2.3$, H6 α), 3.08 (1H, m, H4), 3.06 (1H, dd, 13.8, 3.8, H1'b), 3.03 (1H, m, H6 β), 2.90 (1H, dd, $J=13.8, 6.7$,

H1'a), 2.58 (1H, ddd, $J=14.2, 6.2, 3.1$, H3 β), 2.25 (1H, m, H5 β), 1.63 (1H, m, H5 α), 1.61 (1H, dd, $J=14.2, 12.7$, H3 α); ^{13}C NMR in $\text{D}_2\text{O}/\text{CD}_3\text{OD}$ (40:1) at 67.5 MHz δ 180.0s (COOH-2'), 174.2s (COOH-2), 72.3d (C2'), 60.0d (C2), 44.1t (C6), 39.6 (C4), 35.2t (C1'), 34.3t (C3), 29.8t (C5); ESIMS (rel. ext): 248 [24, (M-H) $^-$], 195 (6), 159 (16), 145 (100), 133 (8), 131 (19), 89 (31), 87 (8), 59 (9); FABMS (rel. ext): m/z 250 [25, (M+H) $^+$], 204 (15), 162 (22), 128 (48), 82 (98). HRFABMS (matrix: thioglycerol): obsd. (M+H) $^+$ m/z 250.0749 ($\text{C}_9\text{H}_{15}\text{NO}_5\text{S}$, $\Delta +0.5$ mmu).

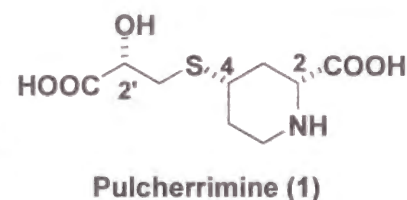


Fig. II-1. A structure of pulcherrimine.

Preparation of Boc derivative 2

Pul (1, 1.8 mg, 0.007 mmol) was converted to triethylamine (TEA) salt by treating with 0.5 mL of TEA and 0.05 mL of H_2O twice. The salt in 0.5 mL of TEA/MeOH (1:1) was treated with $(\text{Boc})_2\text{O}$ (5.0 mg, 0.030 mmol). The mixture was warmed to 50°C and stirred for 24 hr, and the reaction mixture was evaporated to afford a Boc derivative 2 (2.5 mg) as a yellow oil. 2: ^1H NMR in CD_3OD at 270 MHz δ 4.50 (1H, brs, H2), 3.76 (1H, m, H6 α), 3.35 (1H, m, H6 β), 1.40 [9H, s, $-\text{C}(\text{CH}_3)_3$].

Preparation of dimethyl ester derivative 3

A mixture of the Boc derivative 2 (1.25 mg, 0.0036 mmol), DMF (0.5 mL), MeI (1 μL , 0.016 mmol) and NaHCO_3 (5 mg, 0.06 mmol) was stirred overnight at room temperature. The reaction mixture was neutralized with 0.05 N HCl, and extracted with diethyl ether. The ether layer was purified by column chromatography on silica gel (Wakogel C-300) with hexane/EtOAc (1:1) to afford a dimethyl ester 3 (0.2 mg). 3: ^1H NMR in CD_3OD at 270 MHz δ 4.65 (1H, brs, H2), 4.40 (1H, dd, $J=6.2, 4.2$ Hz, H2'), 3.80 (3H, s, $-\text{OCH}_3$), 3.72 (3H, s, $-\text{OCH}_3$).

Preparation of monoacetate 4

The dimethyl ester 3 (0.2 mg) was acetylated with pyridine (100 μL) and acetic anhydride (100 μL) at room temperature for 7 hr. The reaction mixture was evaporated to afford the monoacetate 4 quantitatively. 4: ^1H NMR in CD_3OD at 270 MHz δ 5.21 (1H, dd, $J=7.1, 4.9$ Hz, H2'), 2.16 (3H, s, $-\text{OCOCH}_3$).

Results and Discussion

Pul (1) had a molecular formula of $\text{C}_9\text{H}_{15}\text{NO}_5\text{S}$ as determined by HRFABMS [m/z 250.0749 (M+H) $^+$, $\Delta +0.5$ mmu] and ^{13}C NMR data. The ^1H NMR spectrum (Fig. II-2) displayed three methines [δ_{H} 4.19 (1H, dd, $J=6.7, 3.8$ Hz), 3.08 (1H, m), and 3.64 (1H, dd, $J=12.7, 3.1$)] and eight nonequivalent methylene signals [δ_{H} 3.49 (1H, ddd, $J=13.1, 4.2, 2.3$), 3.06 (1H, dd, 13.8, 3.8), 3.03 (1H, m), 2.90 (1H, dd, $J=13.8, 6.7$), 2.58 (1H, ddd, $J=14.2, 6.2, 3.1$), 2.25 (1H, m), 1.63 (1H, m), and 1.61 (1H, dd, $J=14.2, 12.7$)]. The ^{13}C NMR spectrum (Fig. II-3) together with a DEPT experiment revealed that Pul (1) contained $3 \times \text{CH}$ (δ_{C} 72.3, 60.0, and 39.6) and $4 \times \text{CH}_2$ (δ_{C} 44.1, 35.2, 34.3, and 29.8), in addition to two carbonyl carbons (δ_{C} 180.0 and 174.2). Pul (1) was stable in the 6N HCl solution at 110°C , suggesting that 1 had the absence of peptidic and/or esteric bonds. Therefore, two carbonyl carbons were both carboxyl groups. Interpretation of the COSY, HMQC and HMBC spectra led to partial structures **a** and **b** (Fig. II-4). Chemical shift for C2' ($\delta_{\text{H}}/\delta_{\text{C}}$ 4.20/72.3) and an HMBC cross peak between H2' and a carboxyl carbon at δ 180.0 led to unit **a**. The chemical shifts of C2 and C6 were typical for nitrogen-substituted methine and methylene carbons, respectively. Interpretation of the COSY spectrum starting from an nitrogen-bearing methine at δ 3.64 (H2) led to connectivities from H2 to H6. HMBC cross peaks between H6 α / C2, and H2 and H3 α / a carboxyl carbon at δ 174.2 resulted in unit **b**. The remaining 32 mass unit in the FAB mass spectrum corresponded to one sulfur atom, whose presence was evident from HRFABMS of pseudomolecular ion at m/z 250. An HMBC cross peak between H1' and C4 (partial structure **c**) as well as chemical shifts for C1' ($\delta_{\text{H}}/\delta_{\text{C}}$ 2.90 and 3.06 / 35.2) and C4 (3.08 / 39.6) revealed connectivity between units **a** and **b** through a sulfide bond to establish the gross structure of 1, 4-(2'-carboxy-2'-hydroxy-ethylthio)-2- piperidinecarboxylic acid.

Chemical transformation was carried out to confirm the proposed structure by the procedure shown in Scheme II-2. The presence of two carboxyl groups were confirmed by production of the dimethyl ester upon treatment with MeI under a basic condition (Bocchi *et al.*, 1979a and 1979b) after protection with Boc_2O . Two singlet methyl signals were observed at δ 3.82 and 3.75 in the ^1H NMR spectrum. A hydroxyl group on C2' was acetylated with Ac_2O / pyridine, which was evident from deshielded proton signals (δ 5.21).

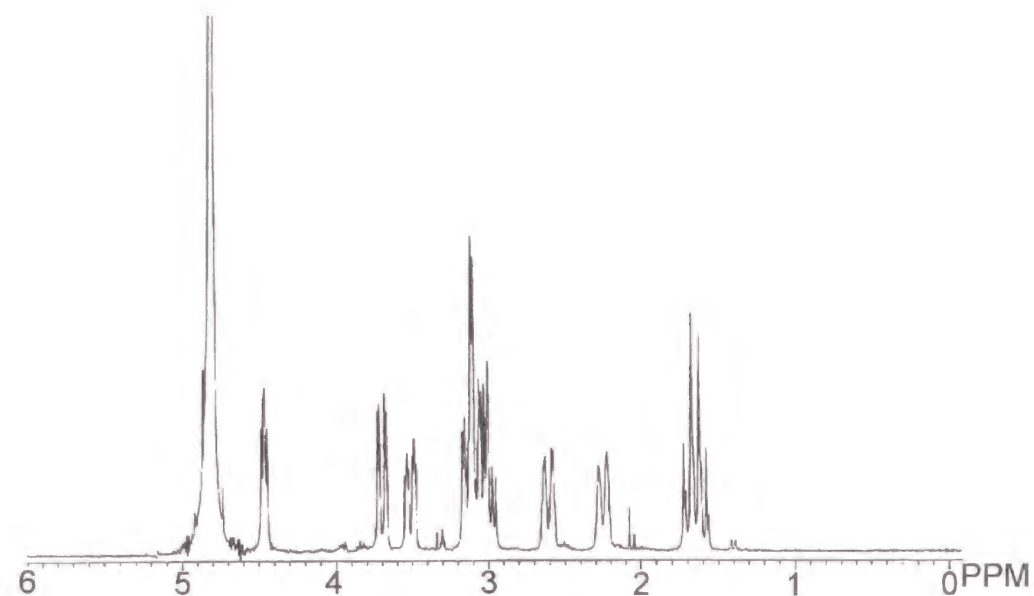


Fig. II-2. ^1H NMR spectrum of pulcherrimine [270 MHz, in D_2O - CD_3OD (40:1), internal standard: CD_3OD]

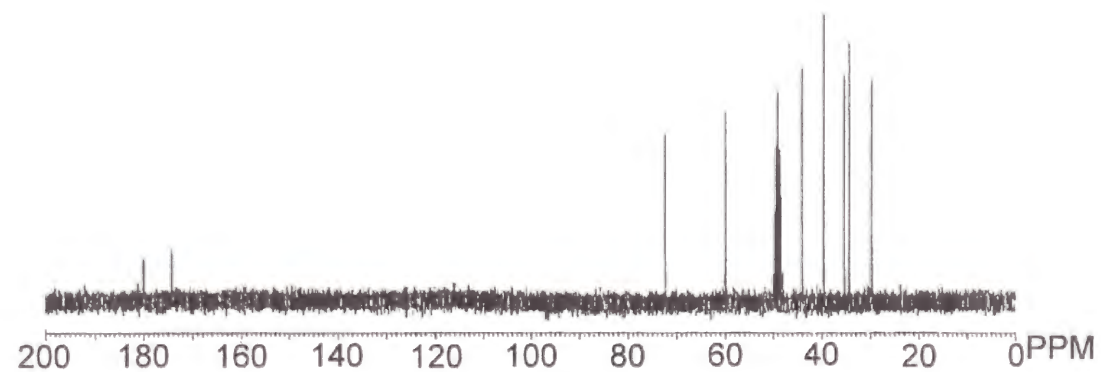


Fig. II-3. ^{13}C NMR spectrum of pulcherrimine [67.5 MHz, in D_2O - CD_3OD (40:1), internal standard: CD_3OD]

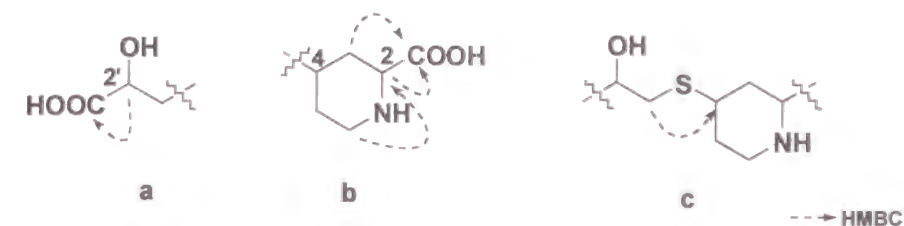
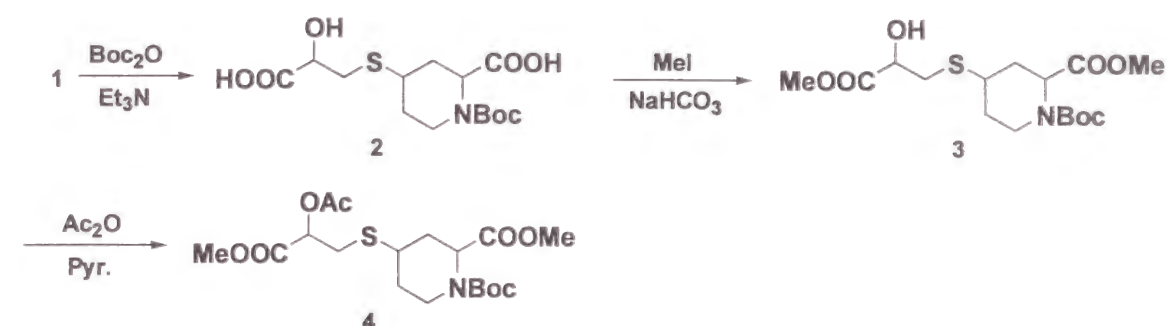


Fig. II-4. Partial structures and HMBC correlations.

Scheme II-2



II-3. Stereochemistry

The planar structure of Pul (**1**) was elucidated as 4-(2'-carboxy-2'-hydroxy-ethylthio)-2-piperidinecarboxylic acid (Section II-2). Pul has three asymmetric carbon, C2', C2, and C4. Therefore, it is thought that there are 8 stereoisomers of 4-(2'-carboxy-2'-hydroxy-ethylthio)-2-piperidinecarboxylic acid.

In this section, the stereochemistry of Pul was determined by NOE experiments, chiral HPLC analysis and modified Mosher method.

Materials and Methods

Chiral HPLC analysis of pipecolic acid

Pul (**1**, 2.0 mg) was hydrogenolyzed at 60°C in the presence of Raney Ni in H₂O for 2 days. The reaction mixture was then evaporated to dryness and dissolved in 5% aq MeCN containing 2 mM CuSO₄. HPLC analysis was carried out with a Shimadzu SPD-10Avp equipped with UV-VIS detector on SUMICHIRAL OA-5000 (4.6 × 150 mm) with 5% aq MeCN containing 2 mM CuSO₄ (detection, UV 254 nm; flow rate, 1.0 mL/min). Retention times: standard L-pipecolic acid (4.667 min), D-pipecolic acid (6.650 min) and product from **1** (7.300 min). Absolute stereochemistry of pipecolic acid obtained from **1** was assigned as D.

Preparation of dimethyl ester derivative, **3**

A mixture of Pul (**1**, 2.4 mg, 0.0096 mmol) and HCl-MeOH reagent (1 mL) was heated at 100°C for 2 hr. The mixture was evaporated to afford a dimethyl ester derivative **5** (2.9 mg). The dimethyl ester derivative **5** was converted to TEA salt by treating with 0.4 mL of TEA and 0.2 mL of MeOH twice. The salt in 0.5 mL of TEA/MeOH (1:1) was treated with (Boc)₂O (3.27 mg, 0.015 mmol). The mixture was warmed to 50°C and stirred for 4 hr, and the reaction mixture was evaporated to dryness. The residue was dissolved in hexane/EtOAc (1:1) and the solution was purified by column chromatography on silica gel (Wakogel C-300) with hexane/EtOAc (1:1) to afford a dimethyl ester **3** (1.8 mg).

Preparation of (R)- and (S)-MTPA esters

(R)- and (S)-MTPA esters of **3** were prepared according to the reported procedures (Dale and Mosher, 1973). To a solution of **3** (0.9 mg) in CH₂Cl₂ (300 μL) and pyridine (300 μL) was added (R)-(-)- or (S)-(+)-MTPA chloride (ca.10 μL), and the solution was stirred at room temperature for 8 hr. The reaction mixture was partitioned between CH₂Cl₂ and 10% KHSO₄. The organic phase was dried (MgSO₄) and purified by column chromatography on silica gel 60 (Katayama Kagaku, 70-230 mesh) with hexane/EtOAc (1:1) to afford a pure sample of the (R)- (1.0 mg) or (S)-MTPA ester (0.5 mg) of **3**.

Results and Discussion

The NOESY cross peaks observed between H4/H2 and H4/H6β and coupling constants indicated their axial orientation, while a W-coupling between H3β and H5β suggested their equatorial relationships. Therefore, the piperidine ring was in a chair conformation having an ethylthio group on C4 and a carboxyl group on C2 as shown in Fig. II-5. To determine the

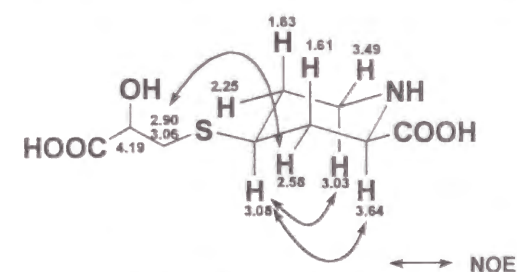


Fig. II-5. A NOE correlations for **1**.

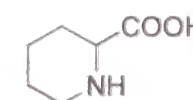
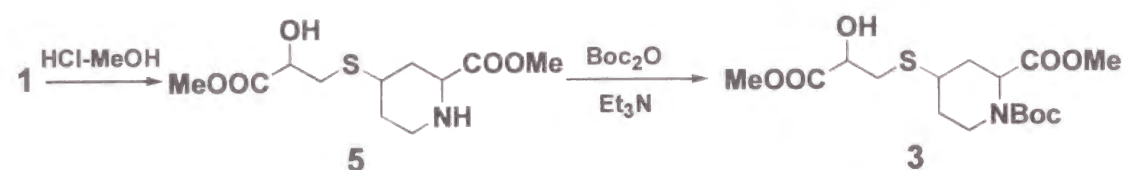


Fig. II-6. A planar structure of pipecolic acid.

configuration of the C2 position, **1** was hydrogenolyzed on Raney Ni in H₂O at 60°C to afford pipecolic acid (Fig. II-6). Chiral HPLC analysis disclosed that the stereochemistry of pipecolic acid was D. The stereochemistry at the C2' position of **1** was determined to be *S* by the modified Mosher method (Dale and Mosher, 1973; Ohtani *et al.*, 1991; Kamiyama *et al.*, 1995; Shin-ya *et al.*, 1995) using the (R)- and (S)-ester of **3** (Scheme II-3). Thus, H1'a and H1'b appeared at upper field in the (R)-ester than in the (S)-ester ($\Delta\delta$, +0.025 ppm and +0.128 ppm, respectively. $\Delta\delta = \delta_S - \delta_R$). While, -OCH₃ (at C3') appeared at upper field in the (S)-ester than in the (R)-ester ($\Delta\delta$, -0.042 ppm). Accordingly, the absolute stereochemistry of **1** was 2'S, 2R, and 4S.

Scheme II-3



CHAPTER III

Determination of pulcherrimine in the green sea urchin gonads

The preceding chapter describes isolation and structure elucidation of pulcherrimine (Pul), 4S-(2'-carboxy-2'S-hydroxy-ethylthio)-2R-piperidinecarboxylic acid from the mature ovaries of the green sea urchin. However, so far there is no determination method to inspect for this substance, other than a sensory test.

In the present study, to develop a method for determining the Pul content in the sea urchin gonads, dimethylaminoazobenzenesulphonyl chloride (Dabs-Cl) was used as a labeling reagent and satisfactory separation was obtained by HPLC (Lin and Chang, 1975; Chang *et al.*, 1981 and 1982). By this method, the Pul content in the gonads of the green sea urchin collected off Iwaki, Fukushima Prefecture in November 1998 was determined.

III-1. Examination of a method for analysis

The preceding Chapter reports that Pul is a novel sulfur-containing amino acid and has an imino group in its structure. Many derivatization reagents for amino acids such as ninhydrin (Spackman *et al.*, 1958), *o*-phthalaldehyde (OPA) (Roth, 1971; Benson and Hare, 1975), dimethylaminoazobenzenesulphonyl chloride (Dabs-Cl) (Lin and Chang, 1975, Chang *et al.*, 1981 and 1982), dimethylaminonaphthalenesulfonyl chloride (Dansyl) (Bayer *et al.*, 1976), phenylisothiocyanate (PITC) (Muramoto *et al.*, 1978), fluorescein isothiocyanate (FIPC) (Muramoto *et al.*, 1984), etc., were examined for HPLC method. Ninhydrin and OPA are widely employed as a colorimetric reagent and a fluorometric reagent, respectively. However, the ninhydrin derivative of Pul was not spectrometrically sensitive enough and OPA did not react with an imino group. Dabs-Cl which is often used as a colorimetric reagent in HPLC analysis for imino acids, amino acids and amines proved suitable for HPLC analysis because of its high sensitivity and the good separation of the Dabs-Cl derivatives (Lin and Chang, 1975; Chang *et al.*, 1981 and 1982).

In the present study, a method for analysis of Pul was examined, using Dabs-Cl as a labeling reagent.

Materials and Methods

Green sea urchin

The green sea urchins were collected off Iwaki, Fukushima Prefecture in November 1998. The sex of all individuals was readily identified from the oozed gametes (Fuji, 1960).

Sample preparation

Gonads from individuals were separately homogenized with an equal volume of distilled water, then the homogenate was heated at 100°C for 12 min. After being cooled, the homogenate was centrifuged at 1700×g for 10 min. The precipitate was re-extracted twice with two volumes and an equal volume of distilled water in a similar manner as above. The extracts were combined in the volumetric flask and filled up to 10 mL with distilled water.

Standard pulcherrimine

Pul as a standard was isolated from the mature ovaries of the green sea urchin by the same method as described in Chapter II.

Reagents

Dabs-Cl was purchased from Tokyo Kasei Co. MeOH (HPLC Grade), EtOH (HPLC Grade), MeCN, AcOH and sodium bicarbonate were purchased from Wako Pure Chemical Co. Dabs-Cl solution was prepared by dissolving 1.3 mg of Dabs-Cl in 2 mL of MeCN, and this solution was kept in -20°C until use.

Dabsylation of amino acids

Dabsylation was performed according to Lin and Chang (1975). Fifty micro liters of each specimen solution were dissolved in 100 µL of 0.1 M sodium bicarbonate (pH 9.0). After 100 µL of Dabs-Cl solution was added to each solution, the mixture was heated at 70°C for 10 min. Then the mixture was evaporated to dryness and subsequently the residue was dissolved in 2 mL of 70% EtOH. The solution was filtered with a 0.5 µm filter membrane (Millipore, Samprep-LCR). Twenty micro liters of the filtrate was injected to the HPLC apparatus.

HPLC analysis

HPLC analysis was performed with a Shimadzu LC-10AT liquid chromatograph equipped with a SPD-10A, UV-VIS detector and a FCV-10AL gradient unit. A HPLC column, YMC pack ODS column, 250 mm × 4.6 mm i.d. was used. As the mobile phase, 40% aq MeOH containing 1% AcOH (A) and MeOH containing 1% AcOH were used. The gradient was 100% to 40% A in 72 min linearly and 40% to 0% A from 72 to 75 min, and kept to 0% A between 75 and 90 min. Flow rate was adjusted at 0.8 mL/min and column temperature was kept at 25°C. The absorbance was monitored at 436 nm.

Results and Discussion

Chromatograms of a Dabs-Pul standard, a dabsylate of extract from an ovary and that from a testis are shown in Fig. III-1. Pul was found to be dabsylated similarly to ordinary amino acids. The dabsylated standard Pul appeared at the retention time of 64.8 min. The other peaks on this chromatogram were considered to be those of by-products derived from the reagents used as mentioned in the previous papers (Lin and Chang, 1975; Chang *et al.*, 1981 and 1982). Dabs-Pul (retention time of 64.5 min) was satisfactory separated from the other dabsylated amino acids extracted from the ovary in the present gradient system. No peak corresponding to Dabs-Pul appeared in the chromatograms for the extract of testis. Chang *et al.* (1982) has developed a complete separation HPLC system for 21 Dabs-amino acids using phosphate buffer containing dimethylformamide (DMF) and acetonitrile containing DMF as gradient solvents. Since the purpose of this study was only to determine Pul, the separation is useful if only Dabs-Pul can be separated from other dabsyl-amino acids. In this sense, a satisfactory separation of Dabs-Pul was performed by the gradient system described in the part of Materials and Methods.

To determine a calibration curve, standard Pul solutions of concentrations ranged from 0 µg/mL to 4 µg/mL were used. As shown in Fig. III-2, a linear correlation ($r=0.994$) was obtained between the Pul concentration (X) and the peak area (Y). This calibration curve was used for the determination of the Pul content of the sea urchin gonads.

The recovery of this method was tested by adding different amounts of Pul ranging from 0.65 to 4.86 mg/100 g to a sea urchin ovary or testis (spiked samples). The spiked samples were extracted and analyzed in the same manner as mention above. Table III-1 shows the

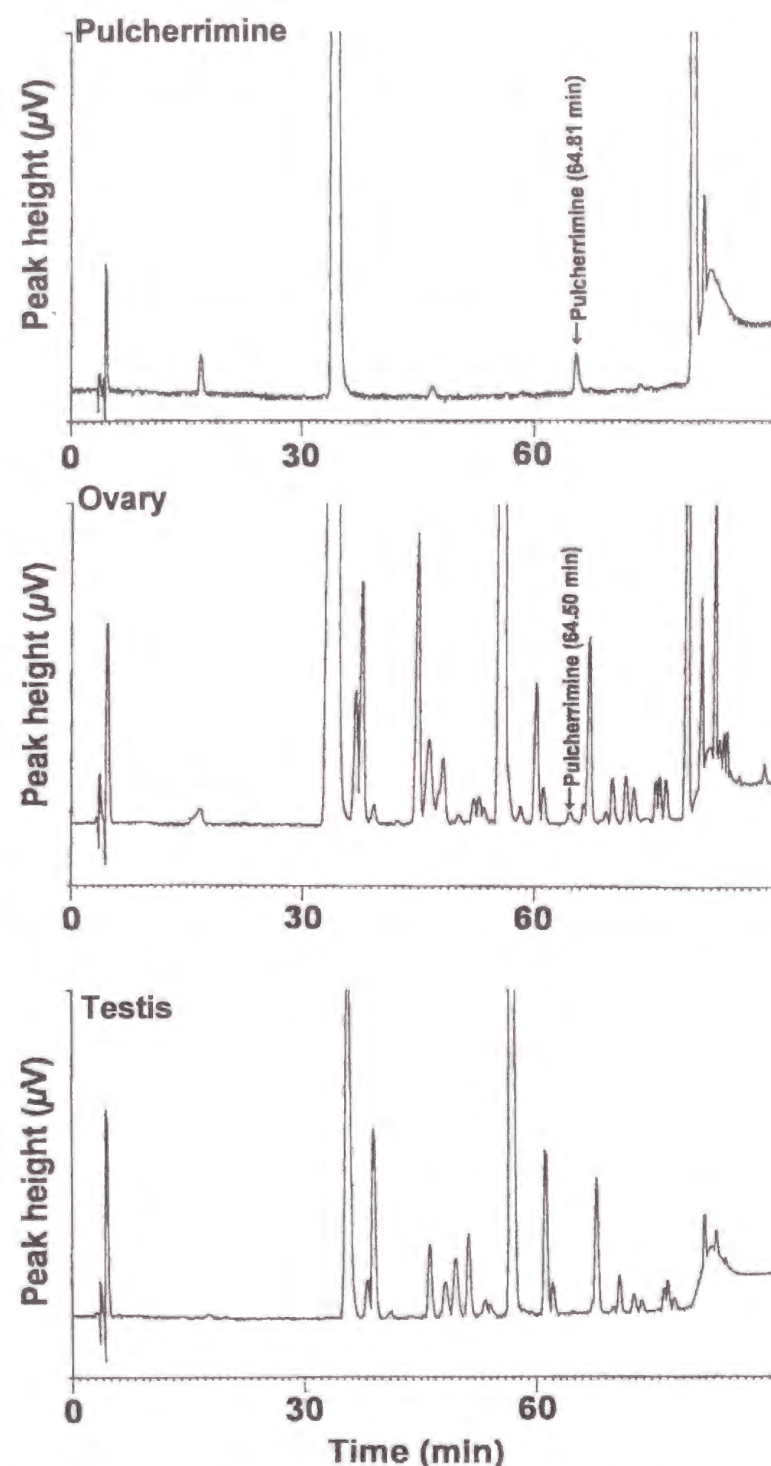


Fig. III-1. HPLC chromatograms of a Dabsyl-pulcherrimine standard, a dabsylate of extract from an ovary and from a testis.
The HPLC column: YMC pack ODS column, 250 mm × 4.6 mm i.d. Mobile phase: A, 40%MeOH containing 1%AcOH; B, MeOH containing 1%AcOH. The gradient was 100% A to 40% A in 72 min, 40% A to 0% A from 72 to 75 min, kept at 0% A between 75 and 90 min. Flow rate was 0.8 mL/min and column temperature was 25°C. The absorbance detection wavelength was 436 nm.

recovery of Pul by this dabsylation-HPLC method. The percent recovery ranged from 86.7% to 91.4%. No significant differences in the recoveries were observed between the ovaries and testes.

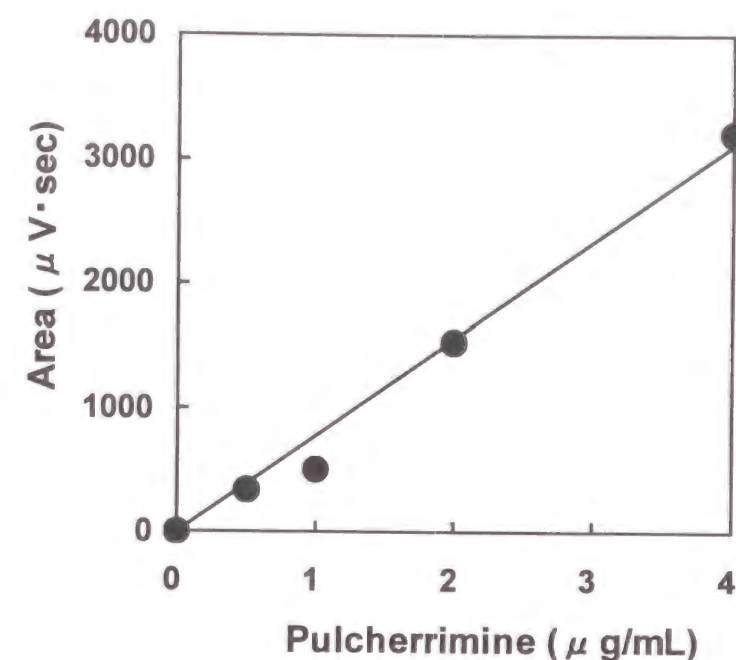


Fig. III-2. Calibration curve for pulcherrimine.

$Y=779.7X$ ($r=0.994$), where X is the pulcherrimine concentration and Y is the peak area.

Table III-1. Percent recovery of pulcherrimine standards from gonads

	Blank (mg/100g)	Amount added (mg/100g)	Amount found (mg/100g)	Recovery [†] (%)
Testes		1.46	1.14	78.1
		1.46	1.47	100.7
		1.46	1.32	90.4
		4.86	4.35	89.5
		4.86	4.51	92.8
		4.86	4.71	96.8
Mean±SD				91.4±7.7
Ovaries	2.04	0.65	2.61	88.0
	1.39	0.65	2.02	96.9
	0.43	0.65	0.95	80.0
	1.00	1.30	2.03	79.2
	1.00	1.30	2.08	83.1
	0.43	1.30	1.64	93.1
Mean±SD				86.7±7.2

[†]Percent recovery = (amount found - blank)/amount added × 100.

III-2. Correlation between bitterness and content

In the preceding section, an analytical method for Pul using Dabs-Cl was developed. The present section describes determination of the Pul content in ovaries and testes of green sea urchins collected at the sea off Iwaki in November 1998 and correlation between bitterness and Pul content.

Materials and Methods

Green sea urchin

The green sea urchins were collected off Iwaki, Fukushima Prefecture in November 1998. The sex of all individuals was readily identified from the oozed gametes (Fuji, 1960).

Sample preparation

Gonads from individuals were separately homogenized with an equal volume of distilled water, then the homogenate was heated at 100°C for 12 min. After being cooled, the homogenate was centrifuged at 1700×g for 10 min. One hundred micro liters of the supernatant were used for taste evaluation and the remainder was transferred to a volumetric flask (10 mL or 20 mL). The influence of using 100 µl of the supernatant on the analysis of Pul content was calculated to be less than 1.25% and was regarded as negligible. The precipitate was re-extracted twice with two volumes and an equal volume of distilled water in a similar manner as above. The extracts were combined in the volumetric flask and filled up with distilled water (Scheme III-1).

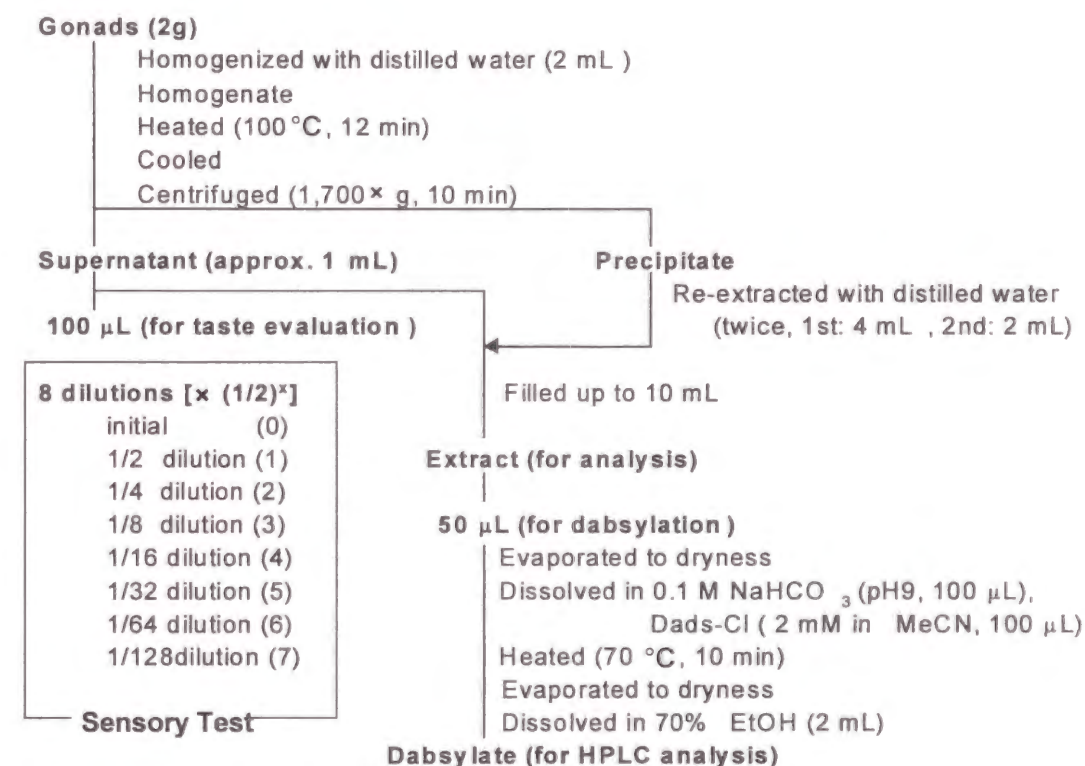
Dabsylation of amino acids and HPLC analysis

Dabsylation of amino acids and HPLC analysis were carried out by the same method as described in the preceding section.

Sensory test

The concentration ranges of extracts for evaluations included eight dilutions from 1 (initial) to 1/128 of dilution which decreased by a factor of 2 and the initial concentration was the original extract. These eight concentration ranges were converted into eight scales of

bitterness. Scales of bitterness were as follows; 0 (initial), 1 (1/2 of dilution), 2 (1/4 of dilution), 3 (1/8 of dilution), 4 (1/16 of dilution), 5 (1/32 of dilution), 6 (1/64 of dilution), 7 (1/128 of dilution). Evaluation was started with the weakest concentration and performed from a weaker to a stronger. The bitterness was defined as the mean value of the scale being converted from the lowest concentration at which the bitterness can be recognized by five subjects.



Scheme III-1

Results and Discussion

Figure III-3 shows the Pul content analyzed for the gonads of 40 sea urchin individuals. Pul content in ovaries ranged from a minimum value of 0.04 to a maximum value of 2.3 mg/100g, and the average was 1.37 ± 0.54 mg/100g ($n=20$). On the other hand no Pul was detected in any of the testes examined ($n=20$). Figure III-4 shows the relationship of bitterness (B) with the Pul content (P) of the 20 individuals analyzed. A linear correlation was discerned between them. The relation can be expressed as an equation: $B=2.08P-0.75$ [$r=0.860$, $n=20$ ($P<0.0001$)].

As to ovary, Pul concentrations obtained were in good agreement with the intensity of bitterness of the sea urchin ovary. On the other hand, no Pul was detected in all the testes examined, and no testis was bitter. These findings indicate that Pul distributes in mature ovaries, but not in mature testes, and that Pul is a bitter component characteristic of the green sea urchin ovaries.

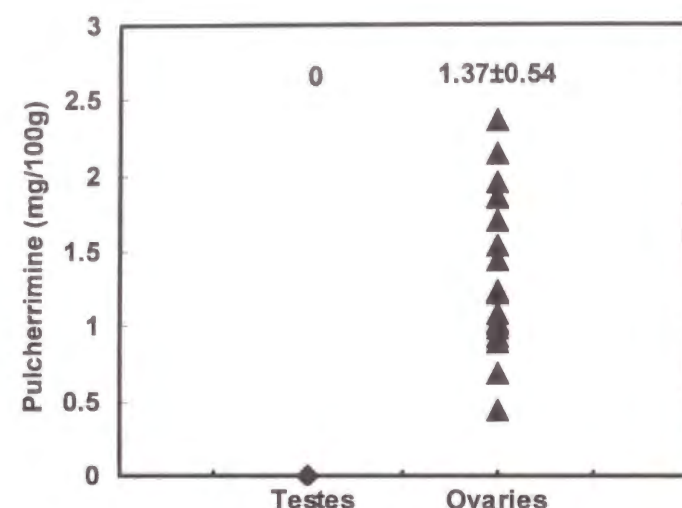


Fig. III-3. Pulcherrimine content of testes and ovaries of the green sea urchin. The data were obtained from 20 individuals of each sex.

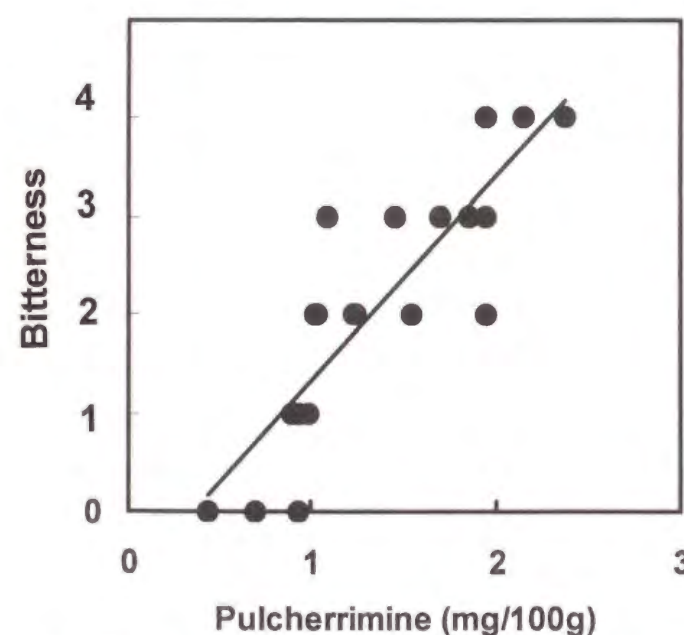


Fig. III-4. Correlation between bitterness (B) and pulcherrimine content (P). $B = 2.08P - 0.75$ [$r = 0.860$, $n = 20$ ($P < 0.0001$)].

III-3. Seasonal changes in content

The previous section (Section I-2) described the result of the tri-monthly examinations in relation to the seasonal changes in the maturity and the frequency of occurrence of bitter gonads of the green sea urchin inhabit in the sea off Iwaki. Accordingly, the seasonal changes in the frequency of bitter ovaries agreed with that in the distribution of mature ovaries.

In the present study, the Pul content of the tri-monthly samples between November 1998 and November 1999 (see Section I-2) was determined.

Materials and Methods

Green sea urchin and sample preparation

The tri-monthly samples were the same samples of the green sea urchin described in Section I-2. Sample preparation was also the same manner as Section I-2.

Dabsylation of amino acids and HPLC analysis

Dabsylation of amino acids and HPLC analysis were carried out by the same method as described in Section III-1.

Results

Twenty specimens were randomly selected from each of mature ovaries and testes and analyzed for Pul content in each month. No Pul was detected in mature testes and they had no bitter taste.

Figure III-5 shows histograms representing the distribution of Pul content among the mature female individuals in each month. Pul content distributions had large variances in all months examined and were different among months. Many individuals with Pul levels more than 0.5 mg/100g were found in November 1998, February 1999 and November 1999. Histograms of February 1999 and November 1999 showed that the mean of Pul content (1.59 and 0.93 mg/100g) was located within the mode column. Also in Nov 1998, the mean Pul content (1.37 mg/100g) located near the mode. Being in contrast, in May and Aug 1999, the mode of each Pul level was at the lowest column, and many mature ovaries with no Pul were

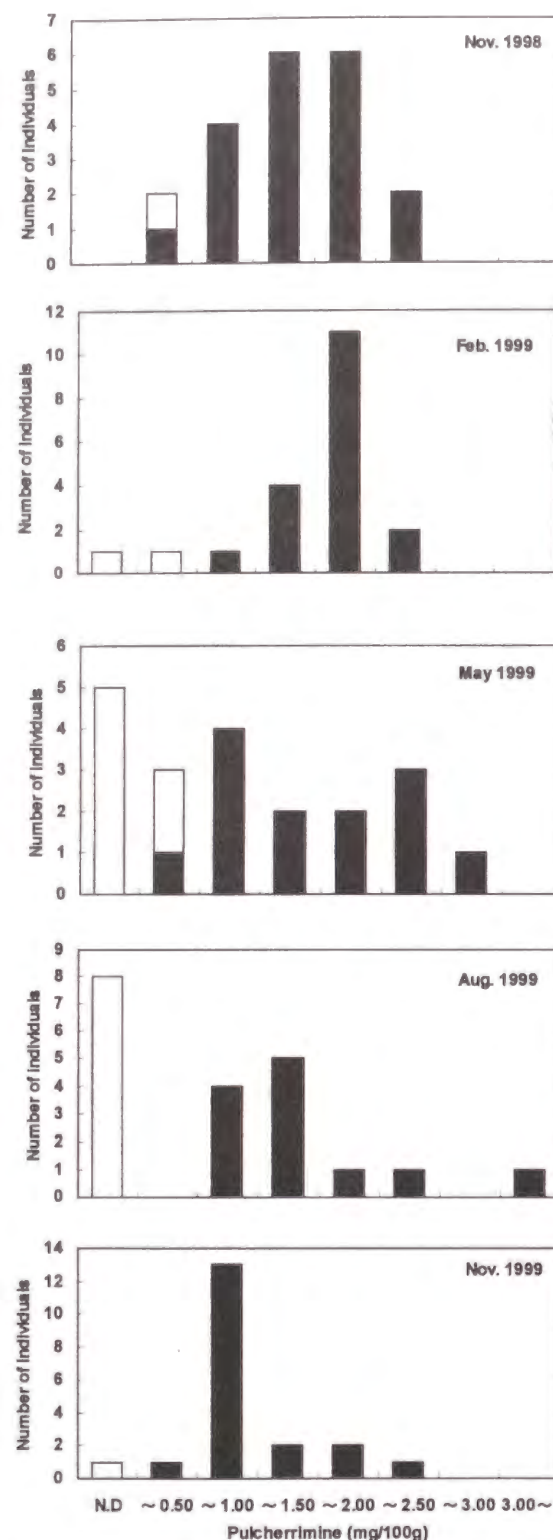


Fig. III-5. Frequency distribution of pulcherrimine contents among mature female individuals in each season. Open and/or solid areas in a bar, indicate non bitter ovaries and bitter ovaries, respectively. N.D means that pulcherrimine was not detected.

found. The distributions were highly skewed for these two months. Some of the non bitter ovaries have been found to have Pul, whose content was less than 0.5 mg/100 g.

Discussion

Pul content distributions of mature ovaries had a large variance in every month and were different among months. Pul levels of mature ovaries were the highest in February, and those of mature ovaries in November 1998 were relatively high. In November 1999, most of the mature ovaries analyzed had more than 0.5 mg/100 g of Pul. On the other hand, in May and August 1999, Pul levels of mature ovaries were relatively low and many non Pul-containing ovaries were found. These results suggest that the amount of Pul is related to the seasonal change of the green sea urchin ovaries.

Analysis of each 20 mature testes in every month showed that Pul was not detected in mature testes. These results are consistent with the preceding Section.

Non Pul containing gonads had no bitter taste. On the other hand, less than 0.5 mg/100 g of Pul was occasionally detected in non bitter gonads. It is probable that the Pul content was lower than its taste threshold level in these gonads.

CHAPTER IV

Sensory tests for pulcherrimine

Most naturally occurring amino acids are considered to have a characteristic taste, and some of them are, of course, important as taste active components in food. Therefore, there are many papers dealing with the taste of amino acids. Solms *et al.* (1969) examined aqueous solutions of L- amino acids (ordinary amino acids) and the corresponding D-form amino acids adjusted to pH 6.0 for their respective tastes, and described that amino acids could be classified into three groups according to their taste qualities. Group 1 consists of amino acids which have no taste at all, or only a barely perceptible taste. Group 2 consists of amino acids with complex taste sensations, which are difficult to evaluate in the pure state. Group 3 comprises of amino acids with distinctive tastes, either bitter or sweet, which were compared quantitatively with caffeine and sucrose solutions, respectively (Solms *et al.*, 1969). Schiffman *et al.* (1976) compiled a list of the taste qualities of amino acids from a number of reports; subsequently, they made a comparison of the taste qualities, and compared the threshold of the taste between corresponding D- and L- enantiomers (Schiffman *et al.*, 1981). Yoshida *et al.* (1969) performed a multidimensional scaling analysis of the taste of amino acids.

As mentioned in Chapter II, pulcherrimine (Pul) was isolated as the substance responsible for the bitter taste to gonads of the green sea urchin, and identified as a novel amino acid. This new amino acid was considered to be classified into the group 3 of Solms' classification (Solms *et al.*, 1969). Therefore, the threshold of this amino acid is of basic importance for the study of the true situation of the occurrence of the bitter green sea urchins, and for studies on the bitter amino acid from the view point of food science.

The present Chapter describes the determination of the threshold values for the detection of and for recognition of Pul. Firstly, the thresholds for Pul in aqueous solution were determined. Secondly, the thresholds, when pulcherrimine is present in an extract of green sea urchin gonads.

IV-1. Detection and recognition thresholds

The threshold for taste active components is generally divided into two categories: the detection threshold and the recognition threshold. The detection threshold is defined as the lowest concentration at which the taste can just be detected, while the recognition threshold is defined as the lowest concentration at which the quality of the taste stimulus can be recognized. The threshold is a statistical concept: a threshold value usually refers to the concentration that is detected or recognized 50% of the time (Bartoshuk, 1978). Sciffman (1981) described the taste qualities and the detection thresholds for 42 kinds of amino acids of L- and D-forms. In the present study, the detection threshold and the recognition threshold for Pul were estimated using a triangle test, which is one of the methods for sensory evaluation of the taste.

Materials and Methods

Subjects

The subjects were 7 males and 3 females from the National Research Institute of Fisheries Science. Their age ranged 20-40 years old.

Stimuli

Pul was isolated from mature green sea urchin ovaries using the same method as described in Chapter II. A series of diluted solutions of Pul ranging from 0.031 to 2 mM was prepared using distilled water.

Procedure

A sensory test was carried out using a triangle test (Schiffman *et al.*, 1981) in which the lick method (Yamaguchi *et al.*, 1994) was incorporated. For this test, 0.1 mL of each solution was placed on a small plastic spoon using a micropipet and the subjects were asked to lick the solutions carefully and judge the taste. Both the detection and recognition thresholds for Pul were determined by trials which began with the weakest concentration and proceeded to progressively stronger concentrations. At each trial, the subjects were presented with a tray on which three unmarked spoons were placed, one of which contained the Pul dilution, and

the other two of which contained only distilled water. After tasting the liquids on each of the three spoons, the subjects judged which spoon contained the stimulus (detection threshold), or which spoon contained the bitter substance (recognition threshold). Percentages of correct judgements for each concentration were plotted and the 50% level of positive responses was defined as the detection or recognition thresholds (Patton and Josephson, 1957). For comparison, both the detection threshold and recognition threshold of quinine sulfate were examined by exactly the same method.

Results

The detection threshold and recognition threshold levels for Pul were determined by tasting seven aqueous solutions differing in the Pul concentration. All the subjects perceived equally the bitterness of Pul, however, the degree of response differed somewhat among individuals. The rates of correct answer for each Pul concentration were plotted as shown in Fig. IV-1 (Patton and Josephson, 1957; Bartoshuk, 1978). The detection threshold and the recognition threshold were estimated as 0.17 mM and 0.34 mM, respectively, based on this plot. As a reference examination, the detection threshold and recognition threshold for quinine sulfate were estimated to be 0.005 mM and 0.019 mM, respectively.

Discussion

The experimental data indicates that the detection threshold for Pul is forty times higher than that of quinine sulfate, which is one of the most typical bitter substances (Pfaffmann *et al.*, 1971). By way of comparison of the detection threshold of Pul with values reported for amino acids by Shiffmann *et al.* (1981), the detection threshold of Pul is approximately the same as that of L-aspartic acid (threshold: 0.182mM) which exhibits a slightly bitter taste. By comparison with L- Val (4.16 mM), L-Leu (6.45 mM) and L-Ile (7.41 mM) which are known as bitter substances in sea urchin gonads (Komata 1964), the detection thresholds for Pul is as small as 1/40 to 1/20 of those such amino acids. When compared with L-threonine which has a slightly bitter taste, the threshold for Pul is as small as 1/100 of that of threonine.

In this study, the lick method was employed using 0.1 mL of test solutions. Yamaguchi and Kobori (1994) described that the detection and recognition thresholds for quinine sulfate

by the lick method with 0.01 mM of the solution were higher than those determined by the whole mouth method with 10 mM of the solution. However, in the case of Pul, the lick simulation with 0.1 mL of test solutions, which was employed in this study, seemed to be a more convenient method than the whole mouth simulation, in view of the facts that Pul tends to leave an aftertaste and there was a restriction on the amount of Pul that could be readily prepared.

Table IV-1. Percentage of correct answers in the triangle tests ($n=10$)

Concentration (mM)	Detection threshold		Recognition threshold	
	Correct answers (%)	Probability	Correct answers (%)	Probability
0.032	30	—	10	—
0.063	20	—	10	—
0.125	40	—	10	—
0.25	60	—	30	—
0.5	70	*	60	—
1	80	**	80	**
2	100	**	100	**

** Binominal tests, $P < 0.05$, * $P < 0.01$, — not significant.

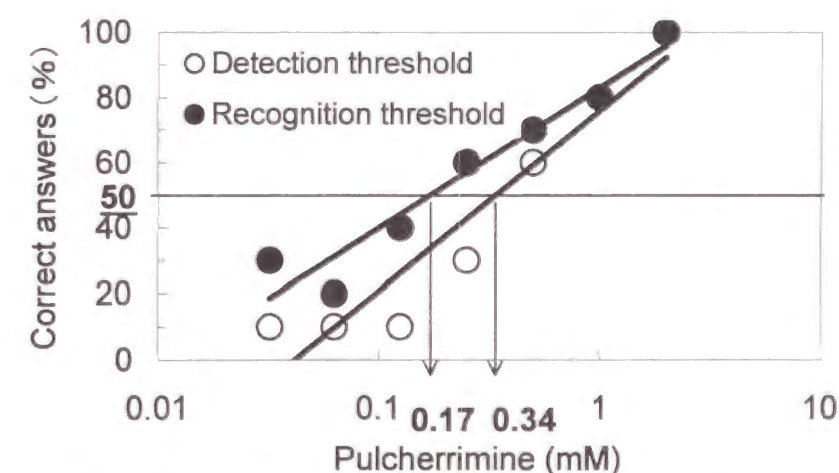


Fig. IV-1. Taste threshold data for pulcherrimine using ten taste panelists.

IV-2. Thresholds in the extracts from sea urchin gonads

In the preceding Section, the detection and recognition thresholds for Pul were estimated using a triangle test to be 0.17 mM and 0.34 mM, respectively. In many cases, the taste of an individual amino acid is complex, and therefore cannot be represented by a single quality dimension such as bitter (Schiffman 1976). Ninomiya *et al.* (1965, 1966) demonstrated the component taste profiles of amino acids. For example, the taste of Val was represented as a component profiles as sweetness (23.4 %), saltiness (0%), sourness (2.2%), bitterness (72.0%) and umami (1.4%). The taste characteristics of each amino acid are greatly influenced by the concentration of their solution, pH and other coexisting substances such as inhibitors and enhancers, etc. Therefore, it must be taken into consideration that some kinds of components extracted from the sea urchin gonads might influence the taste qualities. In the preceding Chapter, it was observed that the Pul content and the degree of the bitterness of mature ovaries (extracts) were significantly correlated to each other.

In the present study, it was examined whether or not the detection and recognition thresholds for Pul are effected by the extractive substances using extracts of mature ovaries, testes, and immature gonads of green sea urchin.

Materials and Methods

Subjects and pulcherrimine

The subjects and pulcherrimine as the specimen are the same as those in the preceding section.

Green sea urchin

Green sea urchins were collected from the coastal sea off Iwaki, Fukushima Prefecture (Fukushima sea urchin) in August 1999 and from the coastal sea off Mikuni, Fukui Prefecture (Fukui sea urchin) in August 2000. The former sea urchins were mature, and sex was readily discernible. Similarly to that mentioned in the preceding Chapter, Pul was detected in most of the mature female individuals collected from the sea off Iwaki, however, in May and August 2000, many female individuals, in which no Pul was detected were found. Such female individuals were used for the examination in this experiment. On the other hand, sea urchins

collected from the sea off Fukui were immature and therefore all the gonads for examinations contained no Pul. The gonads of each sea urchin (male, female, immature) were dissected out and subjected to the extraction with water.

Preparation of test solutions

Nine grams each of male, female and immature gonads were homogenized with 5 mL of distilled water, and the homogenate obtained was heated in boiling water for 12 min. After being cooled, the homogenate was centrifuged at $1700\times g$ for 10 min. The supernatant was transferred to a volumetric cylinder (50 mL) and the residue was re-extracted twice with 10 mL of distilled water in the same manner as above. The extracts were combined in the volumetric cylinder and filled up to 27 mL with distilled water. The extract was divided into two parts: a test solution (9 mL) and a control solution (18 mL). A series of 6 test solutions were prepared by adding Pul to the solution in a range of concentrations of 0.031 to 1mM. To adding the extracts of test solutions and that of control solutions, to be equal to each other as for the concentration of extractive substances in them, the ratio of each extracts to the Pul solution was set to 9:1.

Sensory test and analysis of the data

The procedure of sensory tests and statistical analysis of the data were the same as described in the preceding Section (IV-1).

Results

The detection threshold and recognition threshold for Pul added to the extracts of the green sea urchin gonads were estimated to be 0.25 mM and 0.52 mM in the case of the immature gonad extract; 0.22 mM and 0.46 mM in case of the mature ovary extract; and 0.27 mM and 0.46 mM in case of mature testes extracts (Table IV-2). That is to say, the bitterness of Pul in the gonad extracts was perceived by subjects, regardless of sex and/or the maturity of the sea urchin as far as examined. However, both of the detection and recognition thresholds in each extracts were twice higher than the thresholds observed for distilled water.

Table IV-2. Detection and recognition thresholds for pulcherrimine in the extracts of the sea urchin gonads determined by a triangle test

Concentration (mM)	Immature gonads ^{†1}			Ovaries ^{†2}			Testes ^{†2}		
	Detection threshold	Recognition threshold	Detection threshold	Detection threshold	Recognition threshold	Detection threshold	Detection threshold	Recognition threshold	
	Correct answers (%)	Correct answers (%)	Correct answers (%)	Correct answers (%)	Correct answers (%)	Correct answers (%)	Correct answers (%)	Correct answers (%)	
0.032	10	0	0	30	0	10	10	10	
0.063	30	20	30	30	20	20	10	10	
0.125	40	30	30	30	20	40	20	20	
0.25	40	30	50	50	30	40	20	20	
0.5	50	40	60	60	40	60	50	50	
1	90 ^{†5}	70 ^{†3}	80 ^{†4}	80 ^{†4}	80 ^{†4}	80 ^{†4}	80 ^{†4}	80 ^{†4}	
Threshold (mM) [†]	0.25	0.52	0.22	0.46	0.27	0.46	0.27	0.46	

^{†1}Sea urchin sampled from the sea off Mikuni in Fukui Prefecture.

^{†2}Sea urchin sampled from the sea off Iwaki in Fukushima Prefecture.

[†]The concentration at which the 50% level of the rate of correct answers was obtained.

^{†3} Binominal test, $P < 0.05$, ^{†4} $P < 0.01$, ^{†5} $P < 0.001$.

Discussion

Both of the thresholds for Pul when present in each gonad extract, were higher than those for Pul in distilled water. Many of the subject pointed out that the extracts had a kind of thick taste, and the bitterness of Pul in the extracts was felt to be somewhat vague. The method of Komata (1969) was employed to prepare the extracts in this study. Though the method of Komata (1969) was employed in this study, the ethanol treatment of the extract to remove high-molecular-weight compounds (HMWC) was omitted, because it was found that the ethanol treatment caused the pH to decline and affected the taste quality of the extracts (Murata and Sakaguchi, 1990). The extracts examined might contain various HMWC, glycogen and protein, etc. Glycogen which is contained at a relatively high concentration in green sea urchin gonads shows a distinct body effect by smoothing the taste of the extract (Komata, 1969). Proteins in the extract of sea bream muscle are said to give any additional body to the taste, and gelatin has a masking effect on bitterness. In considering these facts, the extracts examined which contain a large amount of HMWC must mask the bitter taste in some extent. Ming et al. (1999) reported that AMP inhibited behavioral and electrophysiological responses of mice to bitter tastants. As to the effect of HMWC and extractive components on the taste of the extracts of the sea urchin gonads, further study is necessary.

CHAPTER V

Behavioral responses to pulcherrimine using the CTA paradigm in C57BL/6 and BALB/c mice

Many behavioral studies in mice have been made to clarify possible genetic bases for receptor sensitivities to various taste stimuli. By measuring behavioral preference and aversion thresholds with a two bottle test, prominent strain differences in mice have been found in the taste thresholds to many bitter (Lush, 1981,1982,1984), sweet (Fuller, 1974; Lush, 1989) and umami (Bachmanov *et al.*, 2000) substances, and subsequent genetic analysis on the strain differences in some of bitter sensitivities has been made successfully. Sucrose octaacetate (SOA), is one of such bitter substances whose sensitivities have genetically been analyzed in mice (See reviews; Ninomiya *et al.*,1993 and 1999). Warren and Lewis (1979) found that only CFW/NIH mice show a strong aversion to 10^{-3} to 10^{-6} M SOA among 5 inbred strains tested. Their genetic analysis indicates that the strain difference is determined by a single autosomal gene with the 'taster' allele dominant. Lush (1981) reported that SWR is the only strain to show an aversion to drinking SOA for 31 inbred strains. Genetic analysis using a crossbred strain SWR/Lac (taster) and LVC (nontaster), and the 31 inbred strains, suggested that the gene *Soa* determines the ability of a mouse to taste SOA. Further behavioral and genetic studies suggest that sensitivities to quinine (Qui), raffinose undecaacetate (RUA), copper (II) glycinate (GLB) and cycloheximide (CYX) are controlled by the single genes *Qui*, *Rua*, *Glb* and *Cyx*, which are located on the mouse chromosome 6 (Lush, 1991; Lush *et al.*, 1995). These genes are thought to differentially control receptor mechanisms for each different bitter substance (Lush *et al.*, 1995).

The preceding chapter reported that pulcherrimine (Pul) taste bitter to humans with a detection threshold of 0.17 mM, but not examine receptor mechanisms for Pul. If there are many receptors for bitter substances as speculated in the above-mentioned and recent molecular genetic mouse studies (Chandrashekar *et al.*, 2000), it is possible that receptor mechanism for Pul would differ from those for other bitter compounds. As shown in previous mouse studies, possible differences in receptor mechanisms among bitter substances may appear in strain differences in their behavioral thresholds and qualitative discriminabilities among bitter substances. In order to examine this possibility, in this Chapter the author

compared behavioral responses to Pul in C57BL and BALB mice. To measure both quantitative and qualitative responses to pulcherrimine the author employed the conditioned taste aversion (CTA) paradigm whose method logical basis is described as follows. When animals feel internal malaise after ingestion, they learn the taste or smell of that food, and avoid it in future. CTA applies such behavior in animal experiments. Garcia introduced the CTA paradigm into the laboratory (Garcia *et al.*, 1955) and this technique is now used in many physiological studies (Bures *et al.*,1998). An intraperitoneal injection of LiCl is most widely used as unconditioned stimulus (US) for CTA experiments. An animal is injected with LiCl, which elicits internal malaise or nausea after feeding the solution for conditioned stimulus (CS). Then animals learn the CS-US association and avoid the CS (Nachman, 1963; Archer and Sjoden, 1979). In this study, the number of licks per 10 s were counted to examine the strength of the CTA to each stimulus.

V-1. Behavioral thresholds in C57BL/6 and BALB/c mice

In this section, to examine the difference of sensitivity to Pul between C57BL/6 and BALB/c strains, behavioral thresholds for Pul were estimated using the CTA paradigm.

Materials and Methods

Subjects and stimuli

Subjects were adult male and female mice of the C57BL/6 and BALB/c strains weighing 20-30 g. Three mice were housed together in a cage and received *ad libitum* food and water until the start of the experiments. Cages were placed in a light-, temperature- and humidity-controlled room.

The conditioning stimulus (CS) was 4 mM Pul and the test stimuli (TSs) were 0.03 - 4 mM Pul.

Procedures

The present behavioral experiment was carried out according to the method described by Ninomiya *et al.* (1994). On the first day of training, each animal was placed in a test box

and given free access to distilled water during a 1-hr session from a single drinking tube via a circular window (5 mm in diameter). The tip of a polyethylene tube (1.5 mm inner diameter) was located 2.0 mm outside the window. This arrangement prevented contact of the tip of the tube with the animal's lips. Licks were detected by a lickometer with a photo lick sensor and recorded on a digital recorder. From the second to the fifth day, the training session time was reduced from 1 hr to 30 min. During this period, the animal was trained to drink distilled water on an interval schedule, consisting of 10-sec periods of presentation of the distilled water alternated with 20-sec inter-trial intervals, resulting in 30-50 trials during the 30-min session. On the sixth day, each animal was given access to 4 mM Pul during the interval schedule for more than 20 trials, and then given an intraperitoneal injection of LiCl (230 mg/kg) to induce gastrointestinal malaise. The control mice drank distilled water before the LiCl injection. The seventh day was a recovery period, but the training of drinking distilled water for 30 min was still carried out on this day. On the eighth, ninth and tenth days, the number of licks for each 0.03-4 mM Pul solution and distilled water given by each animal was counted during the first 10 sec after the animal's first lick. On each test day, the first test stimulus given to the animal was distilled water. Then Pul solutions of 6 different concentrations were then tested in a descending order. The mean number of licks across the three test days was obtained in each mouse.

Data analysis

The aversion threshold for Pul in each animal was determined as the concentration at which the number of licks per 10 sec was significantly lower than that for distilled water (*t*-test, $P < 0.05$).

Results and Discussion

Figure V-1 shows the number of licks per 10 sec (mean \pm SE) for distilled water and Pul at 6 different concentrations obtained from the C57BL/6 and BALB/c strains after aversions were conditioned in the mice to 4 mM Pul. The aversion threshold for Pul, at which the number of licks was significantly lower than that for distilled water, was 1 mM in C57BL/6 mice, while it was 0.1 mM in BALB/c mice. This fact suggests that the taste sensitivity to Pul is higher in BALB/c than in C57BL/6 mice.

In the preceding Chapter, the human detection threshold for Pul was described to be 0.17 mM. Therefore, the threshold value for Pul in C57BL/6 mice is 5.9 times higher, while that in BALB/c mice is 0.6 times higher than human taste threshold. In ddy mice, the behavioral thresholds for most of amino acids using the two bottle tests was reported to be higher than the human detection threshold by 3-8 times (Kasahara *et al.*, 1987). These discrepancy may be due to the fact that the behavioral threshold is not a direct measure of the recognition threshold, because in mice the neural response thresholds for amino acids, which may be considered as the detection thresholds, were found to be somewhat lower than the behavioral thresholds (Iwasaki *et al.*, 1985).

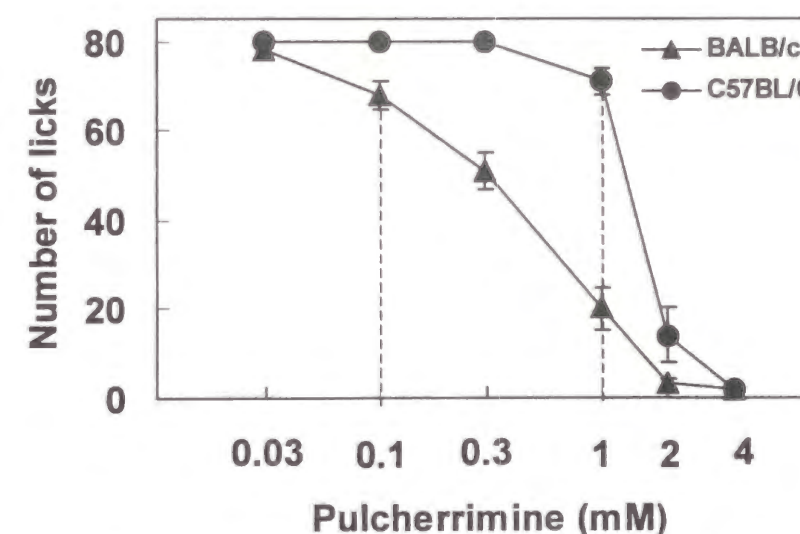


Fig. V-1. The number of licks per 10s (mean \pm SE, $n=5-8$) for 0.03-4 mM Pul after aversions were conditioned in mice to 4 mM Pul. Each dotted line indicates the aversion threshold for pulcherrimine.

V-2. Behavioral responses using the CTA paradigm in C57BL/6 and BALB/c mice

Taste effectiveness and qualitative discrimination of amino acids have been behaviorally examined in mice (Ninomiya *et al.*, 1984; Kasahara *et al.*, 1987; Ninomiya and Funakoshi, 1989) and rats (Pritchard and Scott, 1982) by using the CTA paradigm.

Pul tastes bitter to humans like quinine hydrochloride, valine, brucine, etc. It is thought that many receptor mechanisms contribute to the bitter taste sensitivity. While humans can not discriminate the quality of bitter stimuli as well as mice.

In order to investigate the qualitative similarities and differences between Pul and other taste stimuli, the generalization patterns of a CTA to various compounds were compared between C57BL/6 and BALB/c mice.

Materials and Methods

Subjects and stimuli

The strains of mice were described in the preceding section. Each animal was conditioned to avoid one of 15 compounds and distilled water, forming 15 experimental groups and one control group with 4-6 mice from each strain for each stimuli. Three or four animals in each group were housed together and maintained on *ad libitum* food, but given access to distilled water during the training and testing sessions.

The CS for each of the 15 experimental groups was one of the following; 4 mM Pul, 4 sulfur-containing bitter substances [1 mM 6-n-propylthiouracil (PROP), 1 mM phenylthiourea (PTC), 0.03 M MgSO₄, 0.01 mM quinine sulfate (QH₂SO₄)], 5 non sulfur-containing bitter substances [1 mM sucrose octaacetate (SOA), 0.1 mM denatonium benzoate (Den), 0.03 mM quinine hydrochloride (Qui), 0.03 mM strychnine (Str), 0.1 mM brucine (Bru)], a bitter amino acid [0.1 M L-valine (Val)], a sulfur-containing amino acid [0.1 M L-methionine (Met)], the four basic tastes [0.3M sucrose (Suc), 10mM HCl, 0.1M NaCl, 0.1M MSG]. The CS for the control group was distilled water. These solutions were also used as TSs. The 6 bitter amino acids [0.1 M L-leucine (Leu), 0.1 M L-isoleucine (Ile), 0.1 M L-phenylalanine (Phe), 0.1 M L-arginine (Arg), 0.1 M L-lysine hydrochloride (LysHCl), 0.03 M L-tryptophan (Trp)] were used as TSs.

Procedures

The procedure of the training, CTA and testing was the same as in the preceding section. In this experiment, the number of licks for each of 16 TSs including the CS and distilled water given by each animal was counted during the first 10 s after the animal's first lick.

Data analysis

The strength of the CTA to each stimulus was expressed as a percent suppression according to the formula:

$$\% \text{ suppression} = [1 - (\text{licks}/10 \text{ sec of experimental group}) / (\text{licks}/10 \text{ sec of control group})] \times 100.$$

Cluster analysis was performed with the statistical package SPSS for Windows, version 10.0J. Intercluster similarity was measured using the Pearson's correlation and cluster analysis was performed according to the single linkage method.

Results

CTA generalization pattern

Figure V-2 shows the patterns of suppression of licking across 21 TSs after aversion was conditioned in the C57BL/6 (A) and BALB/c (B) mice to Pul. The solid columns indicate that the number of licks after conditioning was significantly smaller than that in the control animals (*t*-test, $P < 0.05$). Percent suppression for the CS of Pul in the two strains of mice was more than 90%, indicating that the CS was almost equally effective cues. Pul generalized significantly (*t*-test, $P < 0.05$) to PROP, PTC, MgSO₄, QH₂SO₄, SOA and Den in C57BL/6. While in the BALB/c mice, Pul was significantly (*t*-test, $P < 0.05$) generalized to PTC, QH₂SO₄, and SOA. It was commonly observed in the two strains of mice that Pul was weakly (not significantly) or was not generalized to the four basic taste substances (Suc, MSG, NaCl, HCl) and other amino acids.

In order to examine the groupings of the 16 stimuli, a dendrogram for the C57BL/6 (A) and the BALB/c (B) mice, as shown in Fig. V-3, was drawn according to a hierarchical cluster analysis. In the figure, the dotted line in each dendrogram indicates the 1% significance level of correlation (0.605). At this significance level the 16 stimuli were roughly divided into 9 groups in both strains of mice. The 9 groups were composed as follows; (1) Pul, QH₂SO₄, PTC, PROP and MgSO₄, which were the sulfur-containing bitter substances, and SOA, Den

and Str, (2) Bru, (3) Met, (4) Val, (5) Qui, (6) HCl, (7) NaCl, (8) Suc and (9) MSG. In this classification, the generalization patterns between almost all the pairs of stimuli within each group (intragroup) were significantly positively correlated with each other ($P < 0.05$), and those among all pairs of the different groups (intergroup) were not, suggesting the possibility that the mice roughly discriminate among tastes of the corresponding 9 groups of stimuli, in the two strains of mice. As exceptions, no significant positive correlations were found in each intragroup pair between Pul and Den; PROP and PTC, Den and QH_2SO_4 ; SOA and PTC; MgSO_4 and PTC, Den and QH_2SO_4 ; Str and PTC and Den in the C57BL/6 mice, and between PTC and PROP, MgSO_4 , QH_2SO_4 and Str; SOA and PROP; MgSO_4 and Str in the BALB/c mice. A significant correlation ($P < 0.05$) was observed in each intergroup pair between MSG and Str, and Qui and MgSO_4 in the C57BL/6 mice, and between HCl and PTC, Qui and Str and PROP, Bru and SOA, Met and PTC, and Val and Str in the BALB/c mice.

Discussion

Komata *et al.* (1965) elucidated the taste effects of the extractive components from sea urchin gonads, and showed that Val, Leu and Ile contributed to the bitter taste. In the preceding studies, it was found from HPLC analysis and sensory tests that the bitter taste of mature sea urchin ovaries was caused mainly by Pul and not other bitter amino acids (Val, Leu and Ile). Further findings in taste quality of Pul have been made in the present behavioral study in mice using the CTA paradigm.

The generalization patterns to the TSs after conditioning with Pul were observed a difference between C57BL/6 and BALB/c mice. These generalization patterns indicate that Pul may taste bitter to mice and its taste quality differs from amino acids. Thus this fact strongly supports that Pul causes an extraordinary and unacceptable bitter taste in the sea urchin ovaries.

In the two strains of mice, Pul highly generalized to PTC. PTC shows a striking bimodality of the taste threshold in humans (Fox, 1931; Snyder, 1931; Blakeslee, 1932). The threshold concentrations of 'taster' and 'non-taster' for PTC are 0.025 mM and 3 mM, respectively (Kalums, 1971). In the preceding study (Section V-1), it suggested that the taste sensitivity to Pul is higher in BALB/c mice than in C57BL/6 mice. The sensitivity to PTC and also PROP were ascertained to be controlled by a single autosomal gene *Ptc* which locates on

human chromosome 5 (Snyder, 1931, Reed *et al.*, 1999). Although the location of the mouse gene *Ptc* has not been clarified, the loci around *Ptc* on human chromosome 5 are suggested to be homologous to mouse chromosome 13. From these facts, the human sensitivity to Pul also might possibly differ among individuals and might be possibly controlled by a single autosomal gene.

The hierarchical cluster analysis showed that Pul was grouped with sulfur-containing bitter substances, SOA, Den and Str, that is, it showed behavioral similarities among Pul, sulfur-containing bitter substances, SOA, Den and Str. While, the Pul group was divided from the basic taste groups, i.e., sweet, salty, sour, umami tastants, other amino acid groups and other bitter substance groups. The bitter taste transduction mechanisms on Den and SOA reception involve production of inositol 1,4,5-triphosphate (IP_3) in rat and mouse taste cells (Hwang *et al.*, 1990; Spielman *et al.*, 1994 and 1996). That is to say, Den and SOA activate phospholipase C to accelerate the production of IP_3 . IP_3 acts on the endoplasmic reticulum to release Ca^{2+} which is needed for the release of a transmitter from a taste cell (Kashiwayanagi and Kurihara, 1999). Spielman *et al.* (1994) reported that the production IP_3 in mouse taste tissue is stimulated by Str. From these facts, the transduction mechanism of Pul might utilize the pathway involving augmentation IP_3 .

Adler *et al.* (2000) described the isolation of a novel family of 40-80 divergent G protein-coupled receptors, T2Rs, selectively expressed in subsets of taste receptor cells of the tongue and palate epithelium. Recent genetic studies provide new information regarding the taste receptor and transduction mechanisms. Several mechanisms are thought to be operative for the bitter taste of Pul. Future studies are necessary to clarify the receptor and transduction mechanisms of Pul.

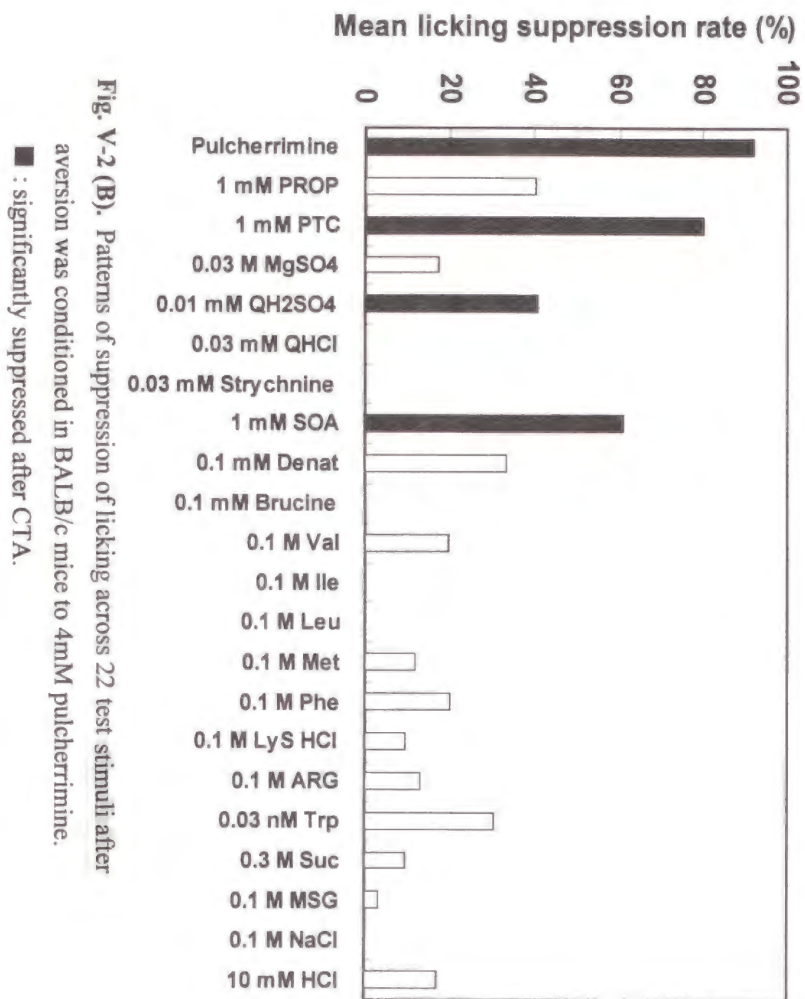


Fig. V-2 (B). Patterns of suppression of licking across 22 test stimuli after aversion was conditioned in BALB/c mice to 4mM pulcherrimine.

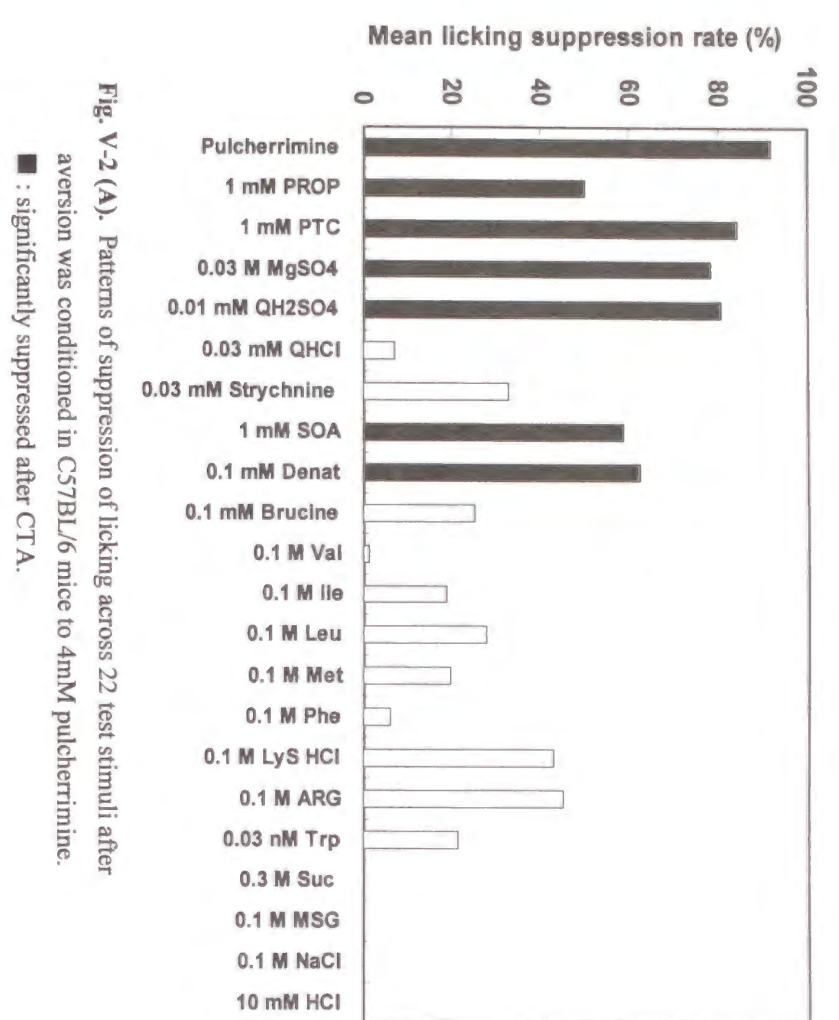


Fig. V-2 (A). Patterns of suppression of licking across 22 test stimuli after aversion was conditioned in C57BL/6 mice to 4mM pulcherrimine.

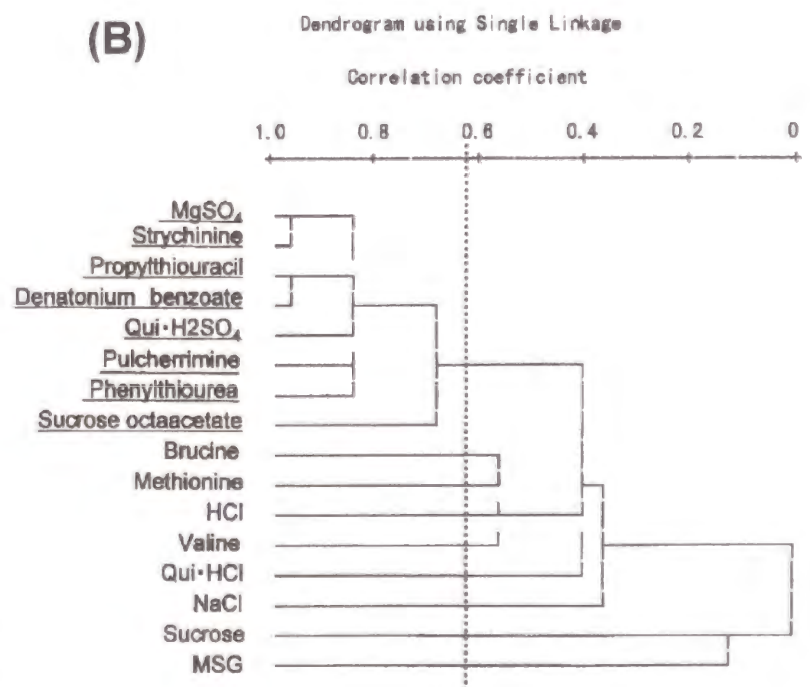
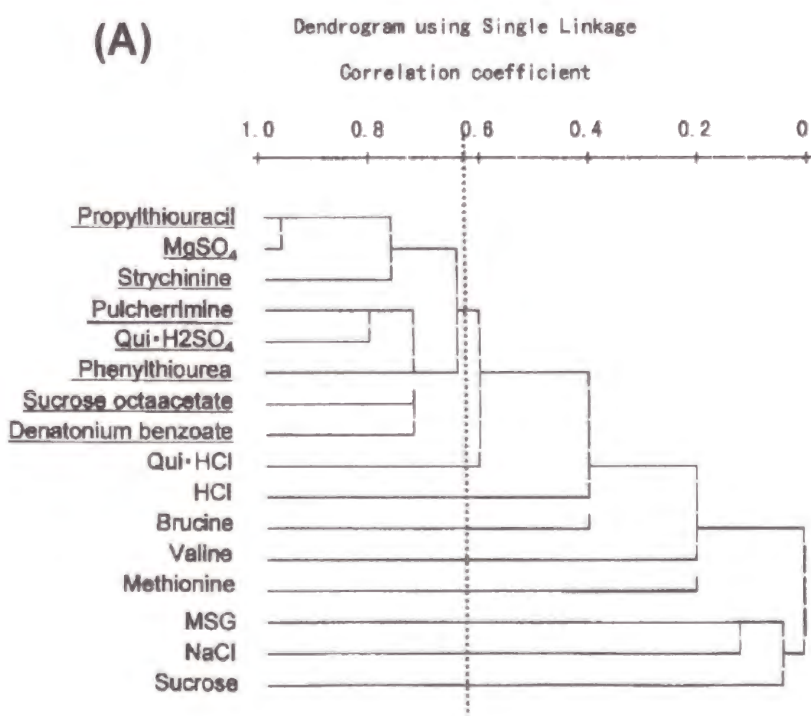


Fig. V-3. Clustering of 16 test stimuli in C57BL/6 (A) and BALB/c (B) mice obtained from a hierarchical cluster analysis. Dotted lines indicate 1% significant level of Pearson's correlation coefficient. Eight underlined letters [in (A) and (B)] indicate to belong in the same group.

CONCLUSION

Green sea urchin is hardly the target for fisheries in the Tohoku area, for example, Fukushima Prefecture because of the bitter-taste of their gonads. This study was undertaken for the purpose of efficient use of the green sea urchin.

Since, in a preliminary experiment, the bitterness was found to be specific to mature ovaries, a bitter substance was isolated from the mature ovaries, and the structure was determined to be 4S-(2'-carboxy-2'S-hydroxy-ethylthio)-2R-piperidinecarboxylic acid, being a novel sulfur amino acid. This substance was named pulcherrimine (Pul) after the scientific name of green sea urchin.

It was found that the GI values showed a considerable variation among the mature specimens in each season, and mature individuals of which ovaries taste bitter were found in all seasons by the tri-monthly examinations. Therefore, the reproductive cycle seems to be unclear and the mature stage extends over a long term in this sea area. These facts may be due to the relatively low water temperature. From these findings, it was concluded that the presence of mature individuals of which ovaries include Pul in all seasons is the major reason for that green sea urchins are hardly utilized for food in the Iwaki area. For the achievement of efficient utilization of this species of sea urchin, their reproductive cycle which varies with individuals must be firstly taken into consideration, although an important question remains still open as to the formation and/or accumulation mechanisms of Pul in the mature ovary of the sea urchin.

The results of the sensory tests confirmed that Pul is the bitter principle present in the green sea urchin ovaries. Furthermore, the bitterness was observed to be weakened by high-molecular-weight compounds (HMWC) present in the extracts of the green sea urchin gonads. However, a further study in relation to the effects of HMWC and extractive components on the bitter taste of Pul is necessary.

The aversion thresholds for Pul in C57BL/6 and BALB/c mice suggested that the taste sensitivity of Pul was higher in BALB/c mice than in C57BL/6 mice. The generalization patterns indicated that Pul may taste bitter to mice and its taste quality differs from bitter amino acids. The hierarchical cluster analysis showed that Pul was grouped with sulfur-containing bitter substances so far known. Further detail studies on the taste receptor and the transduction mechanism of bitter tastants including Pul should prove to be needed and interesting.

SUMMARY

The present study was undertaken to clarify the bitter substance in the green sea urchin gonads. The substance was elucidated to be a novel sulfur-containing amino acid. It was named pulcherrimine after the scientific name of the green sea urchin, *Hemicentrotus pulcherrimus*. The results of the investigation were described in five Chapters.

'Chapter I' described the results of the preliminary examination of this study. The frequency of occurrence of bitter-tasting sea urchins collected from the sea off Iwaki in Fukushima Prefecture was examined. All of the individuals of which the gonads tasted bitter were found to be mature females. This evidence indicates that bitter-tasting gonads are specific to mature ovaries. Free amino acid contents were not significantly different between ovaries and testes. Thus, the possibility of the participation of the bitter tasting amino acids, i.e. Val, Leu and Ile in the bitterness of the sea urchin ovaries was ruled out.

The relation between the tri-monthly changes in the gonad index (GI) and the frequency of bitter gonads of the green sea urchin at the sea off Iwaki was investigated from November 1998 to November 1999. The mean GI value of mature male and female individuals was lowest in May 1999. However, the GI values showed a large variation among mature specimens in each season, therefore, the maturation process of the sea urchins in the sea off Iwaki may vary among individuals. Immature individuals were found in May, August and November 1999, and their percentages were 20%, 60% and 4%, respectively. However, many mature sea urchins were found in all months when examined. These facts indicate that the reproductive cycle seems to be unclear and the occurrence of the mature stage extends over a long term in this area. More than 95% of the mature ovaries had a bitter taste in November 1998, February 1999 and November 1999. In May and August 1999, 60% of the mature ovaries had a bitter taste. In August 1999, 20% of the immature gonads tasted bitter. From these results, the seasonal change in the frequency of bitter ovaries agreed well with that in the distribution of mature ovaries.

'Chapter II' described the isolation of a novel sulfur-containing amino acid from the green sea urchin ovaries, and the elucidation of its structure. This amino acid was named

pulcherrimine (Pul).

Ovaries were extracted with 80% aq EtOH and then 20% aq MeOH. The 80% EtOH extract was partitioned between water and diethyl ether. The aqueous layer and the 20% aq MeOH extract were combined and subjected successively to ODS flash chromatography, gel filtration on Sephadex G-10, MPLC on ODS and RP-HPLC. Thirty milligrams of the bitter principle was obtained from 628 g of the starting material.

Molecular weight and the molecular formula of the bitter compound was determined to be 249 and $C_9H_{15}O_5NS$, respectively, by HRFAB-MS. The structure was elucidated as 4-(2'-carboxy-2'-hydroxy-ethylthio)-2-piperidinecarboxylic acid by 1H and ^{13}C NMR, 1H - 1H COSY, HMBC, and HMQC experiments.

The absolute stereochemistry was determined by NOE experiments, chiral HPLC analysis and the modified Mosher method. Accordingly, the absolute stereochemistry of Pul was 2'S, 2'R, and 4'S.

In 'Chapter III', a method for determination of Pul in sea urchin gonads was devised. This method consists of the formation of dimethylaminoazobenzenesulphonyl chloride (Dabs-Cl) derivatives of Pul (dabsylation), and separation of the dabsylate using RP-HPLC. The dabsylated Pul was analyzed by RP-HPLC using a linear gradient elution consisting of methanol/aqueous/acetic acid and methanol/acetic acid mixture after dabsylation. Linearity of the calibration curve for Pul was extremely high ($r=0.994$) in the tested range from 0 $\mu g/mL$ to 4 $\mu g/mL$.

The Pul content in the ovary and testis of the green sea urchin collected from the sea off Iwaki in November 1998 was analyzed by this method. The mean Pul content in the ovary was 1.37 mg/100g. On the other hand, no Pul was detected in all testes examined. Correlation between the bitterness and the Pul content was statistically significant.

Individuals with a low pulcherrimine content in their ovaries increased from February to August 1999 and decreased from August to November 1999. These results suggest that the amount of pulcherrimine is related to a seasonal change, probably the maturity of ovaries of the green sea urchin.

'Chapter IV' described the result of sensory tests for Pul in relation to the thresholds for detection and recognition.

The detection and recognition thresholds determined by a triangle test were 0.17 mM and 0.34 mM, respectively. Furthermore, by the triangle tests, the thresholds for Pul added to the extracts from the green sea urchin gonads were estimated to be 0.25 mM and 0.52 mM (immature gonads), 0.22 mM and 0.46 mM (ovaries), and 0.27 mM and 0.46 mM (testes), respectively. These findings suggested that the compound exerts remarkable effects of bitter taste on the extracts from both immature and mature green sea urchin gonads.

In 'Chapter V', in order to examine the possibility of differences in receptor mechanisms among bitter substances, behavioral responses to Pul using a conditioned taste aversion (CTA) paradigm in C57BL/6 and BALB/c mice were compared. The behavioral aversion threshold to Pul was determined to be 1.0 mM in C57BL/6 mice and 0.1 mM in BALB/c mice after aversions were conditioned in mice to 4 mM Pul. This fact indicated that taste sensitivity to Pul is higher in BALB/c than in C57BL/6 mice.

Generalization patterns across various bitter and other taste stimuli in the two strains of mice suggested that Pul may taste similar to sulfur-containing bitter compounds, but different from any other bitter amino acids and even L-methionine. A hierarchical cluster analysis showed that Pul was grouped with sulfur-containing bitter substances, and SOA, Den and Str.

ACKNOWLEDGMENTS

The author is deeply indebted to Prof. Morihiko Sakaguchi, Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, for his guidance and encouragement during the present study.

The author is greatly obliged to Prof. Shiro Konagaya, Department of Home Economics, Kokugakuin Tochigi Junior College, for critical reading of the manuscript. The author is grateful to Prof. Yuzo Ninomiya, Section of Oral neuroscience, Graduate School of Dental Science, Kyushu University, for his valuable suggestions and useful comments on the behavioral experiments and Dr. Noriko U. Sata, Department of Chemistry, Faculty of Science and Technology, Keio University, for her contribution to the structure determination.

The author is also grateful to Prof. Michio Shigei, Department of Applied Biology, Faculty of Textile Science, Kyoto Institute of Technology, for the species identification, and Dr. Tatsuya Unuma, National Research Institute of Aquaculture, for his help in the histological experiments. The author is obliged to Mr. Tatsuya Yamamoto, Fukushima Fishery Office, and Mr. Hiroo Yamada, Fukui Prefectural Fisheries Experimental Station, for their help in the sample collection.

The author is indebted to Dr. Jun-ichi Nakazoe and Dr. Ichiro Oohara, National Research Institute of Fisheries Science, Dr. Masahito Yokoyama, Japan International Research Center for Agricultural Sciences, Dr. Masakatsu Yamazawa, Japan Sea Fisheries Research Institute and Dr. Kazuki Shinohara, National Food Research Institute, for their helpful suggestion and discussions. The author wishes to thank Dr. Masaki Kaneniwa and Dr. Ruji Kuwahara, for their assistance and encouragement.

REFERENCES

- Adler E., Hoon M.A., Mueller K.L., Chandrashekar J., Ryba N.J.P. and Zucker C.S. (2000) A novel family of mammalian taste receptors. *Cell*, **100**, 693-702.
- Agatsuma Y. (1992) Annual reproductive cycle of the sea urchin, *Hemicentrotus pulcherrimus* in southern Hokkaido. *SUISANZOSHOKU*, **40**, 475-478. (in Japanese)
- Archer T. and Sjoden P.-O. (1979) Positive correlation between pre- and postconditioning saccharin intake in taste-aversion learning. *Animal Learning and Behavior*, **7**, 144-148.
- Bachmanov A.A., Tordoff M.G., Beauchamp G.K. (2000) Intake of umami-tasting solutions by mice: a genetic analysis. *J. Nutr.*, **130**, 935S-941S.
- Bartoshuk L.M. (1978) The psychophysics of taste. *Am. J. Clin. Nutr.*, **32**, 1622-1627.
- Bax A., Aszalos A., Dinya Z. and Sudo K. (1986) Structure elucidation of the antibiotic desertomycin through the use of new two-dimensional NMR techniques. *J. Am. Chem. Soc.*, **108**, 8056-8063.
- Bayer E., Grom E., Kaltenegger B. and Uhmman R. (1976) Separation of amino acids by high performance liquid chromatography. *Anal. Chem.*, **48**, 1106-1109.
- Benson J.R. and Hare P.E. (1975) *o*-Phthalaldehyde: fluorogenic detection of primary amines in the picomole range. Comparison with fluorescamine and ninhydrin. *Proc. Natl. Acad. Sci. USA*, **72**, 619-622.
- Blakeslee A.F. (1932) Genetics of sensory thresholds: taste for phenyl-thio-carbamide. *Proc. Nat. Acad. Sci. U.S.A.*, **18**, 120.
- Bocchi V., Casnati G., Dossena A. and Marchelli R. (1979a) Esterification of amino acids and dipeptides under mild conditions; Part I: via phase transfer catalysis. *Synthesis*, 957-961.
- Bocchi V., Casnati G., Dossena A. and Marchelli R. (1979b) Esterification of amino acids and dipeptides under mild conditions; Part II: via sodium salts. *Synthesis*, 961-962.
- Bures J., Bermudez-Rattoni F. and Yamamoto T. (1998) *Conditioned Taste Aversion, Memory of a Special Kind*. Oxford University Press, Oxford.
- Chandrashekar J., Mueller K.L., Hoon M.A., Adler E., Feng L., Guo W., Zucker C.S. and Ryba N.J.P. (2000) T2Rs Function as bitter taste receptors. *Cell*, **100**, 703-711.
- Chang J.Y., Knecht R. and Braun D.G. (1981) Amino acid analysis at the picomole level.

- Application to the C-terminal sequence analysis of polypeptides. *Biochem. J.* **199**, 547-555.
- Chang J.Y, Knecht R. and Braun D.G. (1982) A complete separation of dimethylaminoazobenzenesulphonyl-amino acids. Amino acid analysis with low nanogram amounts of polypeptide with dimethylaminoazobenzenesulphonyl chloride. *Biochem. J.*, **203**, 803-806.
- Dale J.A. and Mosher H.S. (1973) Nuclear magnetic resonance enantiomer reagents. Configurational correlations via nuclear magnetic resonance chemical shifts of diastereomeric mandelate, *o*-methylmandelate, and α -methoxy- α -trifluoromethylphenylacetate (MTPA) esters. *J. Am. Chem. Soc.*, **95**, 512-519.
- FAO FISHERY STATISTICS-catches and landings (1995), Vol. 80, Italy.
- Fox A.L. (1931) Taste blindness. *Science*, **73**, 14.
- Fuji A. (1960) Studies on the biology of the sea urchin. I. Superficial and histological gonadal changes in gametogenic process of two sea urchins, *Strongylocentrotus nudas* and *S. intermedius*. *Bull. Fac. Fish., Hokkaido Univ.*, **11**, 1-14.
- Fuke S. and Konosu S. (1991) Taste-active components in some foods: a review of Japanese research. *Physiol. Behav.*, **49**, 863-868.
- Fuller J.L. (1974) Single-locus control of saccharin preference in mice. *J. Hered.*, **65**, 33-36.
- García J., Kimmeldorf D. and Koelling R.A. (1955) Conditioned aversion to saccharin resulting from exposure to gamma radiation. *Science*, **122**, 157-158.
- Hashimoto Y. (1965) Taste-producing substances in marine products. In *the technology of fish utilization* (Edited by Kreuzer R.), pp.57-61, Fishing news Ltd., London.
- Hoshikawa H., Takahashi K., Sugimoto T., Tuji K. and Nobuta S. (1998) The effects of fish meal feeding on the gonad quality of cultivated sea urchins, *Strongylocentrotus nudus* (A. AGASSIZ). *Sci. Rep. Hokkaido Fish. Exp. Stn.*, **52**, 17-24.
- Hwang P.M., Verma A., Bredt D.S. and Snyder S.H. (1990) Localization of phosphatidylinositol signaling components in rat taste cells: Role in bitter taste transduction. *Proc.Nat. Acad. Sci. U.S.A.*, **87**, 7395-7399.
- Ito S., Shibayama M., Kobayakawa A. and Tani Y. (1989) Promotion of maturation and spawning of sea urchin *Hemicentrotus pulcherrimus* by regulating water temperature. *Nippon Suisan Gakkaishi*, **55**, 757-763 (in Japanese).
- Iwasaki K., Kasahara T. and Sato M. (1985) Gustatory effectiveness of amino acids in mice: behavioral and neurophysiological studies. *Physiol. Behav.*, **34**, 531-542.
- Kalmus H. (1971) Genetics of taste. In *Handbook of Sensory Physiology* (Edited by Beidler, L. M.), Vol. 4, Part 2, pp 75-101, Springer-Verlag, Berlin-Heidelberg.
- Kamiyama T., Umino T., Itezono Y., Nakamura Y., Sato T. and Yokose K. (1995) Sulfobacins A and B, novel von Willebrand factor receptor antagonists. II. Structural elucidation. *J. Antibiotics*, **48**, 929-936.
- Kasahara T., Iwasaki K. and Sato M. (1987) Taste effectiveness of some D- and L-amino acids in mice. *Physiol. Behav.*, **39**, 619-624.
- Kashiwayanagi M. and Kurihara K. (1999) Receptor and transduction mechanisms of bitter substances. *Shinkei Kenkyu no Shinpo*, **43**, 649-657.
- Kawana T. (1938) On the propagation of *strongylocentrotus pulcherrimus*. *Suisan Kenkyu Shi*, **33**, 104-116 (in Japanese).
- Komata Y., Kosugi N. and Ito T. (1962a) Studies on the extracts of "Uni". I. Free amino acid composition. *Bull. Jap. Soc. Sci. Fish.*, **28**, 623-629 (in Japanese).
- Komata Y. and Eguchi H. (1962b) Studies on the extracts of "Uni". II. Nucleotides and organic bases. *Bull. Jap. Soc. Sci. Fish.*, **28**, 630-635 (in Japanese).
- Komata Y., Mukai A. and Okada Y. (1962c) Studies on the extracts of "Uni". III. Organic acids and carbohydrates. *Bull. Jap. Soc. Sci. Fish.*, **28**, 747-750 (in Japanese).
- Komata Y. (1964) Studies on the extractives of "Uni". IV. Taste of each component in the extractives. *Bull. Jap. Soc. Sci. Fish.*, **30**, 749-756 (in Japanese).
- Lin J.K. and Chang J.Y. (1975) Chromophoric labeling of amino acids with 4-dimethylaminoazobenzene-4'-sulfonyl chloride. *Anal. Chem.* **47**: 1634-1638.
- Lush I.E. (1981) The genetics of tasting in mice. I. Sucrose octaacetate. *Genet. Res.*, **38**, 93-95.
- Lush I.E. (1982) The genetics of tasting in mice. II. Strychnine. *Chem. Senses*, **7**, 93-98.
- Lush I.E. (1984) The genetics of tasting in mice. III. Quinine. *Genet. Res.*, **44**, 151-160.
- Lush I.E. (1989) The genetics of tasting in mice. VI. Saccharin, acesulfame, dulcin and sucrose. *Genet. Res.*, **53**, 95-99.
- Lush I.E. (1991) The genetics of bitterness, sweetness and saltiness in strains mice. In *Chemical Senses Vol 3, Genetics of Perception and Communication* (Edited by Wysocki C.J. and Kare M.R.), pp. 227-241. Marcel Dekker, New York.

Lush I.E., Hornigold N., King P., Stoye J.P. (1995) The genetics of tasting in mice VII. Glycine revisited, and the chromosomal location of *Sac* and *Soa*. *Genet. Res.*, **66**, 167-174.

Matsui I. (1966) *The program of the sea urchins*. Japan Fisheries Resource Conservation Association, Tokyo (in Japanese).

Ming D., Ninomiya Y. and Margolskee R.F. (1999) Blocking taste receptor activation of gustducin inhibits gustatory responses to bitter compounds. *Proc. Natl. Acad. Sci. USA.*, **96**, 9903-9908.

Miwa K. (1975) Studies on frozen storage of sea urchin gonad. The preventing method for the development of the pungent taste in sea urchin gonad during the frozen storage and the protecting method for the deformation of the gonad at the thawing time. *Bull. Tokai Reg. Fish. Res. Lab.*, **81**, 185-267 (in Japanese).

Monthly Ocean Report (No. 84) December (1999), Climate and Marine Department Japan Meteorological Agency, Japan.

Muramoto K., Kawauchi H. and Tuzimura K. (1978) Sequence determination of peptide by the combined use of fluorescein isothiocyanate and phenylisothiocyanate. *Agric. Biol. Chem.*, **42**, 1559-1563.

Muramoto K., Kamiya H. and Kawauchi H. (1984) The application of fluorescein isothiocyanate and high-performance liquid chromatography for the microsequencing of proteins and peptides. *Anal. Biochem.*, **141**, 446-450.

Murata M and Sakaguchi M (1990) Influence of the ethanol treatment on pHs of boiled meat extracts of fish, shellfish, and domestic animals. *Nippon Suisan Gakkaishi*, **56**, 1697.

Murata Y., Kaneniwa M., Yamashita Y., Iida H. and Yokoyama M. (1998) Compositions of free amino acids and related compounds in the edible portion of ten salmonid species. *Bull. Natl. Res. Inst. Fish. Sci.*, **11**, 65-73 (in Japanese).

Nachman M. (1963) learned aversion to the taste of lithium chloride and generalization to other salts.

Ninomiya T., Ikeda S., Yamaguchi S. and Yoshikawa T. (1965) Studies on the taste of amino acids. *Rep. 6th Sensory evaluation symposium*, JUSE, 16-37. (In Japanese)

Ninomiya T., Ikeda S., Yamaguchi S. and Yoshikawa T. (1966) Studies on the taste of various amino acids. *Rep. 7th Sensory evaluation symposium*, JUSE, 109-123. (In Japanese)

Ninomiya Y., Higashi T., Katsukawa H., Mizukoshi T. and Funakoshi M. (1984)

Qualitative discrimination of gustatory stimuli in three different strains of mice. *Brain Res.*, **322**, 83-92.

Ninomiya Y. and Funakoshi M. (1989) Behavioral discrimination between glutamate and the four basic taste substances in mice. *Comp. Biochem. Physiol.*, **92A**, 365-370.

Ninomiya Y. and Funakoshi M. (1993) Genetic and neurobehavioral approaches for taste receptor mechanisms in mammals. In *Mechanisms of Taste Transduction* (Edited by Simon S.A. and Roper S.D.), pp.253-272. CRC Press, Boca Raton.

Ninomiya Y., Kajiura H., Ishibashi T. and Imai Y. (1994) Different responsiveness of the chorda tympani and glossopharyngeal nerves to L-lysine in mice. *Chem. Senses*, **19**, 617-626.

Ninomiya Y (1999) Genetic approaches for taste receptor and transduction mechanisms. *Shinkei Kenkyu no Shinpo*, **43**, 674-681.

Ohtani I., Kusumi T., Kashman Y. and Kakisawa H. (1991) High field FT NMR application of Mosher's method. The absolute configurations of marine terpenoids. *J. Am. Chem. Soc.*, **113**, 4092-4096.

Patton S. and Josephson D.V. (1957) A method for determining significance of volatile flavor compounds in foods. *Food Res.*, **22**, 316-318.

Pfaffmann C., Bartoshuk L.M. and McBurney D.H. (1971) Taste psychophysics. In *Handbook of Sensory Physiology* (Edited by Beidler L.M.), Vol. 4, Part 2, pp 75-101, Springer-Verlag, Berlin-Heidelberg.

Pritchard T.C. and Scott T.R. (1982) Amino acids as taste stimuli. II. Quality coding. *Brain Res.*, **253**, 93-104.

Reed D.R., Nanthakumar E., North M., Bell C., Bartoshuk L.M. and Price A.R. (1999) Localization of a gene for bitter-taste perception to human chromosome 5p15. *Am. J. Hum. Genet.*, **64**, 1478-1480.

Roth M. (1971) Fluorescence reaction for amino acids. *Anal. Chem.*, **43**, 880-882.

Schiffman S.S. (1976) Taste of dipeptides. *Physiol. Behav.*, **17**, 523-535.

Schiffmann S.S., Sennewald K. and Gagnon J. (1981) Comparison of taste qualities and thresholds of D- and L-amino acids. *Physiol. Behav.*, **27**, 51-59.

Shin-ya K., Shimizu S., Kunigami T., Furihata K., Furihata K. and Seto H. (1995) A new neuronal cell protecting substance, lavanduquinocin, produced by *Streptomyces viridochromogenes*. *J. Antibiotics*, **48**, 574-578.

- Snyder L.H. (1931) Inherited taste deficiency. *Science*, **74**, 151.
- Solms J., Vuataz L. and Egli R.H. (1965) The taste of L- and D-amino acids. *Experientia*, **21**, 692-694.
- Spackman D.H., Stein W.H. and Moore S. (1958) Automatic recording apparatus for use in the chromatography of amino acids. *Anal. Chem.*, **30**, 1190.
- Spielman A.I., Huque T., Nagai H., Whitney G. and Brand J.G. (1994) Generation of inositol phosphates in bitter taste transduction. *Physiol. Behav.*, **56**, 1149-1155.
- Spielman A.I., Nagai H., Sunavala G., Dasso M., Breer H., Boekhoff I., Huque T., Whitney G. and Brand J.G. (1996) Rapid kinetics of second messenger production in bitter taste. *Am. J. Physiol.*, **270**, C926-C931.
- Summers M. F., Marzilli G. and Bax A. (1986) Complete ^1H and ^{13}C assignments of coenzyme B12 through the use of new two-dimensional NMR experiments. *J. Am. Chem. Soc.*, **108**, 4285-4294.
- Unuma T., Konishi K., Furuita H., Yamamo T. and Akiyama T. (1996) Seasonal changes in gonads of cultured and wild red sea urchin, *Psudocentrotus depressus* SUISANZOSHOKU., **44**, 169-175.
- Wakabayashi K. (1984) *Processing of Experimental Data*. Baifu-kan, Tokyo (in Japanese).
- Warren R.P. and Lewis R.C. (1979) Taste polymorphism in mice involving a bitter sugar derivative. *Nature*, **227**, 77-78.
- Yamaguchi S. and Kobori I. (1994) Humans and appreciation of the umami taste. In *Olfaction and Taste XI* (Edited by Kuruwara K., Suzuki N. and Ogawa H.), pp. 353-356, Springer-Verlag, Tokyo.
- Yoshida M. and Saito S. (1969) Multidimensional scaling of the taste of amino acids. *Jap. Psychol. Res.* **11**, 149-166.